PROTECTIVE EFFECTS OF TWO DEFENCE GENES IN TRANSGENIC PLANTS

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Declaration

I hereby declare that this thesis is my own work except where stated to the contrary. I particularly acknowledge the contributions made by Peter Hughes, Andy Moore, Stuart Craig and James Ridsdill-Smith and these are clearly defined in the text. This thesis is less than 100 000 words in length, and is not substantially the same as any other thesis that has been submitted at any other university.

Julia Charity

Julia A Charity

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Dedication

I dedicate this thesis to the memory of Annette Joy Snow - my dear friend who died suddenly on 28.2.96. Annette made a substantial contribution during her time as Ph.D student in our lab. She demonstrated, for the first time that a bacterial fructosyl transferase could be expressed in transgenic plants. Her work has implications for modifying plant carbohydrates which may lead to more efficient energy utilisation. Annette is sadly missed and will always be remembered.

Abstract

Proteinase inhibitors and thionins are among the proteins thought to have a role in plant defence. Genetic engineering offers an attractive means to increase plant productivity by using these natural defensive agents to improve their resistance to pests and pathogens. With this aim in view, cDNA clones encoding the precursors of a multi-domain proteinase inhibitor from *Nicotiana alata* (Na-PI) (M_r ~43 000) and a β-hordothionin (β-HTH) (M_r ~13 000) from barley, were reconstructed for expression in leaves and transferred by *Agrobacterium*-mediated transformation into tobacco. The Na-PI or β-HTH precursors were processed in transgenic tobacco and accumulated as polypeptides of size M_r ~6000 or M_r ~8500, respectively. The Na-PI cDNA was also transferred to pea and subterranean clover, and in both cases M_r ~6000 polypeptides accumulated in the leaves, indicating that the precursor was also processed in legumes. The *na-pi* and β-*hth* genes segregated as dominant traits and were stably inherited for at least two generations. Transgenic tobacco plants containing the highest amount of Na-PI and β-HTH were cross-fertilised to produce individual plants containing both genes.

Helicoverpa armigera or *H. punctigera* (tobacco and native budworm, respectively) larvae that ingested transgenic tobacco or pea leaves containing Na-PI exhibited higher mortality and slower development relative to control larvae. The effect of Na-PI was dose-dependant and *H. punctigera* appeared more sensitive to Na-PI than *H. armigera*. In transgenic tobacco containing both Na-PI and β-HTH, the protective effect of each defence agent was additive.

Transgenic tobacco containing either Na-PI or β-HTH were significantly more protected against the fungal pathogen, *Botrytis cinerea* (grey mould), than non-transgenic controls. The effect of the two genes was additive, although the contribution of each gene to the protective effect was not equal. β-HTH provided a major increase in resistance, while the addition of Na-PI marginally increased this tolerance.

β-HTH also conferred effective protection against *Pseudomonas solanacearum* (bacterial wilt) infection in transgenic tobacco. Tobacco which contained both Na-PI and β-HTH were also more resistant to *P. solanacearum,* although in this case the effect of the two genes was not additive.

The genetic engineering of plants with proteinase inhibitors or thionins, therefore, has tremendous potential for improving crop productivity by increasing resistance to insects and diseases.

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Chapter One

Introduction

Chapter One

Genetic Manipulation of Plants for Improved Resistance to Pests and Pathogens

1.1 Introduction

The economic and social impact of damage to plants by pests and disease is substantial. At least 37% of all crops worldwide are lost to insects and pathogens, and despite an annual insecticide expenditure of about US $7 billion, 13% are destroyed by insect feeding (Gatehouse and Hilder, 1994). Currently the main methods of controlling insects and fungi involve the integrated use of chemical sprays (Lamb *et al.,* 1992), resistant plant varieties and the management of agricultural practice to include crop rotation and field sanitation. Because chemicals are expensive and can have a negative impact on the environment and encourage the development of resistance in some insects, there is a need to look for alternative methods for the control of pests and pathogens. Such management strategies include new technologies like genetic engineering.

1.2 Genetic manipulation of plant defence

Genetic engineering provides a tool for the introduction of stably inherited genes to improve pest and disease resistance in plants. An advantage of genetic engineering is that genes from other plants, microbes or animals can be inserted into the target plant. Furthermore, resistance is heritable and, therefore, permanent. The main requirement for producing transgenic plants is a reliable regeneration and transformation system. Over 100 plant species are now amenable to transformation and many of these are important food and pasture crops (Panda and Khush, 1995; Siemens and Schieder, 1996). One limiting factor in the application of genetic engineering for plant improvement is the availability of genes that confer suitable pest or disease control characteristics. Strategies for enhancing crop resistance via genetic engineering have been the subject of many recent reviews (Lamb *et al.*, 1992; Cornelissen and Melchers, 1993; Tabe *et al.*,

1993; Gatehouse and Hilder, 1994; Ward *et al.*, 1994). This chapter will discuss insecticidal and antimicrobial molecules and their use in transgenic plants to improve crop productivity. Two specific groups of defence-related proteins: serine proteinase inhibitors and thionins will be the focus of this review.

1.2.1 Using defence-related proteins to improve plant resistance

Plants have evolved many mechanisms to prevent or minimise damage by pests and pathogens. The first barrier is normally passive, for example, the leaves of plants often have hairs or thorns which act as feeding deterrents. Additionally, the waxy cuticle and suberised periderm strengthen and protect the cell walls from damage.

The second line of defence consists of preformed secondary metabolites such as complex polymers like lignin, cellulose, phenolics and callose, as well as glycine-rich proteins and hydroxyproline-rich glycoproteins (cell wall proteins) (Cooper *et al.,* 1987).

Thirdly, in response to pest or pathogen invasion, plants recognise substances of microbial origin, so-called elicitors which induce the production of an array of molecules with protective effects. The elicitors initiate the hypersensitive response (HR); which is an active defence mechanism associated with resistance to numerous pathogens (Kiraly, 1980). Its main feature is the rapid necrosis of plant cells surrounding the infection site of an invading pathogen. HR also results in the accumulation of toxic chemicals (phytoalexins) and an array of antagonist proteins. These so-called defence-related molecules are generally classified according to their mode of action (Bowles, 1990). These defence molecules are assumed to function in the inhibition of pathogen multiplication and spread. For example, some defence molecules act directly as antimicrobial toxins (defensins, lectins, osmotins, zeamatins and thionins), while others are enzymes. These include α-amylase inhibitors, proteinase inhibitors, pathogenesis-related proteins (eg: β-1,3-glucanases, chitinases), ribonucleases, ribosome-inactivating proteins and lipid-transfer proteins. Genes encoding many of these antimicrobial agents have been transferred into plants and their efficacy has been assessed in bioassays (Table 1-2).

1.2.1.1 Plant-derived genes as insecticidal or antimicrobial agents in transgenic plants

In choosing suitable antimicrobial or insecticidal genes as candidates for genetic engineering, plants themselves are a rich source of defence-related molecules. Proteinase inhibitors and thionins are the most important insecticidal and antimicrobial proteins for this thesis, so are discussed fully in sections 1.3 and 1.4, respectively. There are other plant-derived genes which are potentially useful in providing plants with additional protection from pests and pathogens, and a few examples are discussed in this section. Toxic proteins are considered first, followed by examples of enzymes thought to be involved in the production of secondary metabolites with a putative defence role in plants.

Lectins

Lectins have been reported in most flowering plant families as well as a range of algae, fungi and micro organisms. They are abundant in seeds, roots, shoots, bark, tubers, membranes and phloem exudate (see reviews Etzler, 1985; Pusztai, 1991). Lectins are carbohydrate-binding proteins that agglutinate cells of non-immune origin and/or complex with carbohydrates (polysaccharides, glycoproteins, glycolipids) (Goldstein and Hayes, 1978).

The glycopolymer chitin is an important structural component of the cell walls of certain fungi and the exoskeleton and neurotrophic membrane of nematodes and insects. Although lectins were first described as storage proteins, certain chitin-binding lectins have anti-fungal activity, leading to the speculation that they may have a role in defence against pests and pathogens (Etzler, 1985). The best studied are lectins from legumes, cereals and solanaceous plants. For example, wheat germ agglutinin inhibits spore germination and hyphal growth of the fungus *Trichoderma viride* (Mirelman *et al.,* 1975; cited in Ward *et al.,* 1994). In addition, pea seed lectin sprayed onto pea leaves one day before inoculation, prevented the formation of necrotic lesions caused by *Mycosphaerella pinodes* (Lepoivre and Boy, 1983). However these *in vitro* studies

must be evaluated with caution because in some cases the antifungal activity may be attributable to chitinases present in the lectin preparation (Raikhel *et al.*, 1993).

As well as being anti-fungal, there is some evidence that lectins protect plants against insect damage. Lectins from wheat, castor beans and camel's foot tree were lethal to European corn borer when supplied at 2% of the soluble protein in an artificial diet (Czapla and Lang, 1990). In another bioassay with an artificial diet, Shuckle and Murdock (1983) demonstrated that soybean lectin slowed the development of *Manduca sexta* larvae. Bean (*Phaseolus vulgaris*) seed lectin was thought to be responsible for increased mortality of *Callosobruchus maculatus,* (Gatehouse *et al.,* 1984) although this conclusion proved incorrect, when Huesing *et al.,* (1991) showed that it was the α-amylase inhibitor contaminating the lectin preparation which was toxic to *C. maculatus.*

Some lectins, for example those from winged bean, soybean and wheat germ, are limited in their application in crop protection because they can be toxic to mammalian cells (Pusztai, 1991). Pea lectin, however, is insecticidal *in vitro* (Boulter *et al.,* 1990) but is innocuous to mammals because it is broken down in the gut. Transgenic plants accumulating pea lectin to high levels have enhanced resistance to tobacco budworm (Boulter *et al.*, 1990). These tobacco plants were cross-bred with tobacco containing the cowpea trypsin inhibitor, (CpTI) and progeny expressing both genes retarded growth of *Helicoverpa virescens.* The effect of the two genes was additive (Boulter *et al.*, 1990) (Table 1-2).

Other antimicrobial peptides

In addition to lectins, there is a family of small, anti-microbial peptides (AMPs) characterised by a high proportion of basic amino acids and intramolecular cysteine bridges. At least five classes of AMPs have been characterised (Table 1-1). One class, the plant defensins, are 45-54 amino acids and are structurally related to each other as well as resembling insect and mammalian defensins. Plant defensins have eight cysteine residues, an aromatic residue at position 11 and two glycines which are conserved (Broekaert *et al.,* 1995). In general, their 3-dimensional structure consists of a triple-

stranded, antiparallel β-sheet and a single α-helix lying in parallel with the β-sheet (Broekaert *et al.,* 1995).

Table 1-1 Five classes of plant antimicrobial peptides (AMPs)

Class of AMP	Example	Size	Disulphides	Reference
Thionins	Barley endosperm	5 kDa	4	Hernández-Lucas *et al.,* 1986; Ponz *et al.,* 1986
Knottin-type	*Mirabilis jalapa*	4 kDa	3	Cammue *et al.,* 1992
Hevein-type	*Amaranthus caudatus*	3-4 kDa	3-4	Broekaert *et al.,* 1992
Plant defensins	*Raphanus sativus*	5 kDa	4	Terras *et al.,* 1992a and b; 1995; Broekaert *et al.,* 1995
Lipid transfer proteins	Maize and wheat	10 kDa	4	Shin *et al.,* 1995 and Gincel *et al.,* 1994

There is increasing evidence that these peptides may play a role in plant defence; for example, radish antifungal peptides (Rs-AFP2) are released during seed germination, and appear to protect the emerging cotyledon from fungal infection (Terras *et al.,* 1995). Supporting evidence for the role of (Rs-AFP2) in plant protection was obtained when the gene for the antifungal peptide from radish seeds was transferred into tobacco. The transgenic plants were more resistant to *Alternaria longipes* (Terras *et al.,* 1995) (Table 1-2). As well as antimicrobial activity (Terras *et al.,* 1992b; Moreno *et al.,* 1994), other roles have been described. Some plant defensins exhibit α-amylase inhibitory activity (Bloch and Richardson, 1991) and others inhibit protein synthesis *in vitro* (Mendez *et al.,* 1990). Furthermore, these antimicrobial peptides accumulate to high levels in seeds and are rich in sulphur-containing amino acids, which suggests that they may also have a secondary role as storage proteins.

Not all antimicrobial peptides belong to the five classes mentioned in Table 1-1 and aside from seed-specific deposition, antifungal peptides are produced in leaves after wounding (Hedrick *et al.,* 1988), exposure to pathogens (Ryan, 1984; Lamb *et al.,* 1992 and references within) or elicitors (Lawton and Lamb, 1987). Floral organs also are a rich source of proteins with potential antimicrobial properties (Lotan *et al.,* 1989; Atkinson *et al.*, 1993b; Constabel and Brisson, 1995). Some components of the pistil may be

involved in protecting the reproductive tissues from potential pathogens or insects. For example, petunia stigmas contain an active chitinase (Leung, 1992) and in members of the Solanaceae, defence-related molecules in stigmas and styles include 'extensin-like' proteins, a serine proteinase inhibitor, β-1,3-glucanases and chitinases (Atkinson *et al.*, 1993b and cited references). Stress-induced proteins are also likely to have anti-microbial properties (Carr and Klessig, 1989) and are often called 'pathogenesis-related' (PR) proteins. Other antimicrobial peptides with potential for increasing bacterial disease resistance include cecropins which are a family of polypeptides from insect hemolymphs (Boman and Hultmark, 1987; Jaynes *et al.*, 1987). A cecropin isolated from the giant silk moth exhibited potent activity against plant pathogenic bacteria (Nordeen *et al.*, 1992). Other molecules such as lipid-transfer proteins (Terras *et al.*, 1992a), osmotins and polygalacturonase inhibitors (Lamb *et al.*, 1992) also have antifungal activity.

α-*Amylase Inhibitors*

While the physiological role for α-amylase inhibitors (α–AIs) in plants is not known, there is accumulating evidence which suggests they may be involved in protection from insect pests. When α-AIs from wheat and barley were tested against a range of moths, aphids, petatomids, thrips, beetles and stored cereal/grain pests, Gutierrez *et al.*, (1990) concluded that these α–AIs were differentially active against the insects. Effective α-amylase inhibitory activity from wheat was correlated with the presence of a M_r 13 000 polypeptide (Gatehouse *et al.*, 1986). Only wheat fractions containing this molecule inhibited the digestive α-amylases from larvae of *Tribolium confusum* and *Callosobruchus maculatus,* both pests of stored grain (Gatehouse *et al.*, 1986). A recent report describes transgenic peas expressing bean α-AI that were resistant to the pea weevil (Coleoptera) (Schroeder *et al.*, 1995) and extends the previous observation that bean α-AI was active in artificial diets fed to *C. maculatus* (Huesing *et al.*, 1991). The transgenic peas were also resistant to the adzuki bean weevil and cowpea weevil (Shade *et al.*, 1994), which are major pests of stored seed (Table 1-2).

Chitinases and β-1,3-glucanases

These enzymes catalyse the hydrolysis of chitin and β-1,3-glucan, respectively, which are the major components of the cell walls of many fungi and oomycetes. One strategy to increase a plant's resistance to pathogens is to engineer the constitutive expression of defence genes that are normally induced only after pathogen attack. In the first successful application of this approach, both transgenic tobacco and *Brassica napus* expressing a chitinase gene from beans, had increased resistance to seedling rot (Broglie *et al.*, 1991). Furthermore, the simultaneous expression of a chitinase and a β-1,3-glucanase in transgenic tomatoes gave a high level of resistance against *Fusarium oxysporum* (van den Elzen, 1993; Jongedijk *et al.,* 1995) which suggests that these hydrolases act synergystically (Table 1-2).

Ribosome-inactivating-proteins

Ribosome-inactivating-proteins (RIPs) inhibit protein synthesis by specific N-glycosidase modification of 28S rRNA (for review, see Stirpe *et al.*, 1992). This modification is irreversible and renders the ribosome unable to bind elongation factor 2, thereby blocking translation. RIPs do not affect host-plant ribosomes but show various degrees of specificity towards other plant and fungal ribosomes (Stirpe and Hughes, 1989). The use of RIPs in crop protection will depend upon the extent of their toxicity in the target plant cells. One indication of success is that high level expression of a barley RIP in pollen and floral organs of transgenic tobacco had no effect on fertility and the plants were partially resistant to *Rhizoctonia solani* (Logemann *et al.*, 1992) (Table 1-2). RIP purified from barley acted synergistically *in vitro* with chitinase to inhibit the growth of *Trichoderma* and *Fusarium* species (Leah *et al.,* 1991), however this has not been confirmed *in vivo.*

1.2.1.2 Bacterial toxins as insecticidal agents in transgenic plants

Another source of insecticidal genes is a family of genes from *Bacillus thuringiensis* (*B.t.*) which encodes a potent toxin (Hofte and Whiteley, 1989). The toxin is produced as a precursor by sporulating bacteria. The precursor is ingested by insects and proteolytically cleaved to liberate the active toxin. Different classes of *B.t.* crystal proteins have been identified based on their sequence similarity` and specificity with respect to the insects to which they are toxic. For example, 'CryI' toxins are active

against the Lepidoptera order (moths and butterflies), CryII proteins are toxic to Lepidoptera and Diptera (flies), CryIII proteins are toxic to Coleoptera larvae (beetles) and all CryIV proteins are active against mosquito and blackfly larvae. A high level of resistance to caterpillar pests (especially *Helicoverpa* species) has already been achieved in tomato, tobacco, maize and cotton containing *B.t.* CryI proteins (Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987; Perlack *et al.*, 1990; Kumar and Sharma, 1994) (Table 1-2). Total reliance on *B.t.* genes for pest control risks the rapid development of insects resistant to the toxin (Gould *et al.*, 1992) in the same way that resistance to insecticides has evolved amongst *Helicoverpa* species (Daly, 1993). Because this is a major concern, suitable alternative genes for insect resistance are urgently required for the development of pest resistant crops. This thesis is an investigation of other insecticidal molecules which could have the potential to increase insect mortality and restrict growth.

Table 1.2 Evaluation of transgenes with a potential role in plant defence

TRANSGENE	TARGET ORGANISM	PROTECTIVE EFFECT ON TRANSGENIC PLANT	REFERENCE
***LECTINS*:**			
Snowdrop lectin (*Galanthus nivalis*)	Peach-potato aphid (*Myzus persicae*)	Tobacco: significant increase in resistance	Hilder *et al.*, 1995
Snowdrop lectin (*Galanthus nivalis*)	Peach-potato aphid & *Lacanobia oleracea*	Potato: resistance to larvae of both insect pests	Gatehouse *et al.*, 1995
***THIONINS*:**			
Barley α-hordothionin	*Pseudomonas syringae*	Tobacco: decreased lesion size	Carmona *et al.*, 1993b
Wheat α_1-purothionin	*Pseudomonas syringae*	Tobacco: no effect	Carmona *et al.*, 1993b
Barley α-hordothionin	*Clavibacter michiganensis*	Isolated thionin from transgenic tobacco inhibited growth	Florack *et al.*, 1994
Barley α-hordothionin	*C. michiganensis*, *P. solanacearum* & *Xanthomonas campestris* pv. *vesicatoria*	Tobacco: no increased resistance to these bacteria	cited in Florack and Stiekma, 1994
Barley β-hordothionin	Grey mould (*Botrytis cinerea*)	Tobacco: reduction in lesion area	This thesis
Barley β-hordothionin	Bacterial blight (*P. solanacearum*)	Tobacco: significant reduction in plant mortality - minor symptoms	This thesis
***ANTIMICROBIAL PEPTIDES*:**			
Radish seed antifungal peptide Rs-AFP2 - plant defensin	*Alternaria longipes* (foliar fungal pathogen)	Tobacco: lesion areas 7-8 fold smaller than in control plants	Terras *et al.*, 1995
Tabtoxin (from *P. syringae* pv. *tabac*	*P. syringae* pv. *tobacci*	Tobacco: complete resistance - no symptoms	Anzai *et al.*, 1989
Killer toxin (KP4) from *Ustilago maydis* (smut fungus)	Isolates of *Ustilago* sensitive to KP4	Tobacco: leaf disks placed on lawn of fungus prevent growth	Park *et al.*, 1996
Tachylplesin I from Asian horse-shoe crab	Potato soft rot (*Erwinia carotovora*)	Potato: Tubers are slightly more resistant.	Allefs *et al.*, 1996

α-AMYLASE INHIBITORS:			
Bean seed α-amylase inhibitor	Pea weevil *(Bruchus pisorum)* Cowpea weevil (*Callosobrucus maculatus*) & Azuki bean weevil (*C. chinensis*)	Peas: weevil development in T_5 seed was completely inhibited Peas: complete resistance to these bruchid beetles	Schroeder *et al.*, 1995 Shade *et al.*, 1994
ENZYMES:			
Bean chitinase (class I)	Damping off (*Rhizoctonia solani*)	Tobacco and Canola: delay in disease symptoms, better survival	Broglie *et al.*, 1991
Tobacco chitinase (class I)	*Cercospora nicotiana*	*Nicotiana sylvestris:* no enhanced resistance, even at high levels	Neuhaus *et al.*, 1991
Barley ribosome inactivating protein	Damping off (*Rhizoctonia solani*)	Tobacco: some protection, based on plant height	Logemann *et al.*, 1992
Tobacco pathogenesis-related protein 1a (PR-1a)	Black shank disease (*Phytophthora parasitica*) & Blue mould (*Peronospora tabacina*)	Tobacco: significant delay in disease onset	Alexander *et al.*, 1993
Tobacco PR-1	Tobacco mosaic virus	Tobacco: no additional resistance	Cutt *et al.*, 1989; Linthorst *et al.*, 1989
Glucose oxidase (*Aspergillus niger*)	Bacterial soft rot *(Erwinia carotovara* ssp. *carotovora*)	Potato: high level of resistance	Wu *et al.*, 1995
	Potato late blight (*Phytophthora infestans*)	Potato: enhanced resistance, lesion development delayed	Wu *et al.*, 1995
Pear polygalacturonase inhibitor protein	Grey mould *(Botrytis cinerea)*	Tomato: fruit were less infected; i.e., enhanced resistance	Powell *et al.*, 1994
Stilbene synthase (groundnut)	Grey mould *(B. cinerea)*	Tobacco: 80% reduction in disease symptoms	Hain *et al.*, 1990 and cited in Cornelissen and Melchers (1993).
Stilbene synthase (grapevine)	Grey mould *(B. cinerea)*	Tobacco: enhanced resistance	Hain *et al.*, 1993
Bean phenylalanine ammonia-lyase 2	Frogeye disease (*Cercospora nicotianae*)	Tobacco: increased susceptibility - rapid lesion development	Maher *et al.*, 1994
Bacteriophage T_4 lysozyme	Soft rot (*Erwinia carotovora*)	Potato: significant reduction in fungal growth	Düring *et al.*, 1993

B.t. TOXINS			
_B.t_ truncated _B.t_ toxin: CryIAb	Tobacco hornworm (*Manduca sexta)*	Tobacco: highly protected - up to 100% insect mortality	Vaeck *et al.,* 1987
	Tobacco budworm (*Helicoverpa armigera*) & native budworm (*H. punctigera*)	Cotton: high level of protection - low insect survival	Perlack *et al.,* 1990
	Tobacco budworm (*H. armigera*)	Tomato: significant resistance	Reynaerts and Jansens (1994)
	Striped stemborer (*Chilo suppressalis*)& Leaf folder (*Cnaphalocrosis medinallis)*	Japonica rice: plants more resistant - increased insect mortality and reduced biomass	Fujimoto *et al.,* 1993
	Tuber moth (*Phtorimaea operculella*)	Potatoes: leaves and tubers highly resistant	Peferoen *et al.,* 1990
_B.t_ toxin: CryIIA	Colorado potato beetle *(Leptinotarsa decemlineata*)	Potatoes: significant resistance to feeding damage	McPherson *et al.,* 1989
TWO OR MORE TRANSGENES			
Pea lectin and cowpea trypsin PI	Tobacco budworm (*Helicoverpa virescens*)	Tobacco: 2 genes are additive: less leaf damage, reduced biomass	Boulter *et al.*, 1990
Tobacco class I chitinase and/or class I β-1,3-glucanase	*Fusarium oxysporum* f.sp. *lycopersici* race 1	Tomato: both genes act synergistically to enhance fungal resistance	Jongedijk *et al.,* 1995
Barley chitinase, β-1,3-glucanase and RIP	*Rhizoctonia solani*	Tobacco: enhanced tolerance	Jach *et al.,* 1995
Barley β-hordothionin and *Nicotiana alata* proteinase inhibitor (Na-PI)	Tobacco budworm (*H. armigera*)	Tobacco: Effect of two genes is additive, significantly smaller larvae and delayed development	This thesis
β-hordothionin and Na-PI	Grey mould *(Botrytis cinerea)*	Tobacco: major protective effects of β-HTH, minor effect of Na-PI but additively reduced lesion area	This thesis
	Bacterial blight (*Pseudomonas solanacearum*)	Tobacco: improved resistance - only β-HTH is responsible	This thesis

1.3 Serine proteinase inhibitors

Proteins that form complexes with proteases to inhibit their proteolytic activity are found in both animal and plant kingdoms. I will restrict my discussion to plant proteinase inhibitors (PIs) and will cover their characterisation, function and exploitation as defence molecules to minimise crop damage by insect pests.

Proteases can be divided into four mechanistic classes i.e.; serine-, cysteine-, aspartic- and metallo-proteases. Inhibitor families have been found that are specific for each class (Laskowski and Kato, 1980). Most plant proteinase inhibitors (PIs) inhibit serine proteases (for details of these and others, see Leiner and Kakade, 1980; Ryan and An, 1988; Ryan, 1990; Brzin and Kidric, 1995).

1.3.1 Distribution and types of serine PIs

Serine PIs are present in the seeds, leaves and tubers of several members of the Fabaceae (legumes), Poaceae (cereals) and Solanaceae families (Richardson, 1977). They have been categorised into 19 families on the basis of molecular weight, amino acid sequence and enzyme specificity (see reviews; Richardson, 1977; Ryan, 1984; Ryan 1990; Richardson, 1991; Bode and Huber, 1992; Heath, 1994). The PIs belonging to the potato inhibitor families I and II are the most important for this chapter, not only because they are the most well characterised, but they have sequence similarity to the proteinase inhibitor from *Nicotiana alata* (for details of others, see García-Olmedo *et al.,* 1987; Ryan, 1990; Brzin and Kidric, 1995).

Potato tubers contain proteinase inhibitors I and II (pin1 and pin2) which accumulate to levels of 5% of the soluble protein in potato tubers (Richardson, 1977), the level varying with developmental stage (Ryan, 1973; Mitsumori *et al.,* 1994). Similarly, in a wild species of tomato (*Lycopersicon peruvianum* (L.) Mill., LA107), the fruit contains over 50% of its total soluble protein as PIs which rapidly decline during maturation (Pearce *et al.,* 1988). PIs with sequence similarity to potato pin1 and pin2 from tubers, accumulate in wounded potato and tomato leaves (Green and Ryan, 1972; Plunkett *et al.,* 1982;

Graham *et al.,* 1985a; 1985b; Graham *et al.,* 1986). Comparisons between potato pin1 and pin2 are made in Table 1-3.

Table 1-3. Comparison between potato proteinase inhibitors: pin1 and pin2.

	Potato Inhibitor I (pin1)	**Potato Inhibitor II (pin2)**
Protomers	5 protomers; each 8.1 kDa	2 protomers; each 11.5 kDa
Total size	41 kDa	23 kDa
Total number of disulphides	5	10
Inhibitory reactive site	Chymotrypsin	1 chymotrypsin, 1 trypsin*
Reference	Ryan, 1984	Bryant, *et al.,* 1976; Ryan, 1984; García-Olmedo *et al.,* 1987

*variants with two chymotrypsin or two trypsin sites have also been described (Sanchêz-Serrano *et al.,* 1986; Hass *et al.,* 1982).

Other PIs with sequence similarity to the proteinase inhibitor II family of potato and tomato include an auxin-inducible PI in tomato roots (Taylor *et al.,* 1993). The open reading frame contains a putative signal peptide and three trypsin-specific domains (Taylor *et al.,* 1993). The developing shoot apex of tomatoes also contains a polypeptide with significant similarity to the class II PIs from potato and tomato (Brandstadter *et al.,* 1996). Also related to the type II PIs is a stress-induced tobacco leaf proteinase inhibitor (PI-II) with three homologous domains, each with a different (but not yet identified) reactive site (Balandin *et al.,* 1995). Tobacco leaves also contain a trypsin inhibitor (TTI) (Pearce *et al.,* 1993) which are approximately 66% identical to tobacco PI-II (Balandin *et al.,* 1995). Pin2 also has partial sequence identity (~55%) to a proteinase inhibitor precursor from *Nicotiana alata* which has six repeated domains and inhibits trypsin and chymotrypsin (Atkinson, 1992; Atkinson *et al.,* 1993a; see section 1.3.5).

1.3.2 Synthesis and regulation of PIs

The changes in PI levels during maturation of potato tubers (Ryan, 1973; Mitsumori *et al.,* 1994) and tomato fruit (Pearce *et al.,* 1988) indicate that PI synthesis is regulated by developmental control mechanisms. However, PIs are also induced in leaves by

environmental stresses such as wounding or mechanical damage (Green and Ryan, 1972; Plunkett *et al.,* 1982; Graham *et al.,* 1985a; 1985b; Graham *et al.,* 1986). The promoter region of the potato pin2 gene has been studied in detail in transgenic tobacco by fusion of promoter deletions to reporter genes (Thornburg *et al.,* 1987; Keil *et al.,* 1989; 1990). To summarise, the nucleotides -1300 to -195 of the promoter region of the pin2 gene were sufficient for wound-inducible expression and gene activity was enhanced by including 260 bp of the 3' untranslated region (Keil *et al.,* 1990). In addition, 10 nucleotides in the promoter region were found to react with a wound-inducible nuclear protein. The position of these nucleotides was adjacent to an 8 bp sequence which has been found in other elicitor- or light-responsive elements (Palm *et al.,* 1990). Furthermore, the entire chimeric gene was developmentally regulated in a normal manner (Keil *et al.,* 1989), implying that the promoter of potato pin2 controls both wound-inducible and tuber-specific expression and that this regulation may involve cis- and trans- acting factors (Sanchêz-Serrano *et al.,* 1987).

The induction pathway of potato PIs in response to environmental stress is complex and involves a systemic signal that mediates induction of PI genes throughout green, aerial tissues. Because pest and pathogen attack induce such a diverse array of defence responses (also see section 1.2.1) there have been a number of possible long-distance signalling molecules identified and characterised. Elicitors that regulate the expression of wound-inducible PI genes were shown to be oligosaccharides, probably originating from the cell wall (Ryan *et al.,* 1985). Later studies showed that substitutes like chitosan or polygalacturonic acid can also be used to increase the expression of pin2 in detached potato leaves (Sanchêz-Serrano *et al.,* 1986) or in transgenic tobacco (Sanchêz-Serrano *et al.,* 1987). Additionally, three of the octadecanoid precursors of jasmonic acid were powerful inducers of PI synthesis, suggesting they could be candidates for signalling molecules (Farmer and Ryan, 1992). Other suggested signalling molecules include abscisic acid (Peña-Cortés *et al.,* 1989), jasmonates (Farmer *et al.,* 1992), salicylic acid (SA) (Dempsey and Klessig, 1994), an electrical signal (Wildon *et al.,* 1989; 1992; Rhodes *et al.,* 1996) and systemin (Pearce *et al.,* 1991; Schaller and Ryan, 1995). The current view is that while SA is involved in development of systemically acquired

resistance (SAR) (Klessig and Malamy, 1994), there must be other translocatory signals, as SA cannot be transported to other parts of the plant (Métraux *et al.,* 1990). There is good evidence that systemin is the signal molecule which activates PIs in tomatoes and potatoes, not only in leaves which have been damaged by wounding, but in distal, unwounded leaves (Schaller and Ryan, 1995). Systemin is an 18 amino acid polypeptide (Pearce *et al.,* 1991) which is proteolytically processed from a 200 amino acid precursor, prosystemin (McGurl *et al.,* 1992). The evidence that systemin is part of the signalling system for the induction of pin1 and pin2 is that it is a powerful inducer of PI synthesis, it is capable of translocation in the phloem (Narváez-Vásquez, *et al.,* 1995) and constitutive expression of antisense prosystemin in transgenic plants resulted in severe suppression of wound-inducible PI expression (McGurl *et al.,* 1992). After wounding and translocation of systemin throughout the tomato or potato plants, systemin is thought to bind to a specific receptor. One such systemin-binding protein has been identified in tomato plasma membranes (Schaller and Ryan, 1994). Binding of systemin results in the activation of the octadecanoid pathway. This lipid-based signalling pathway eventually leads to the induction of PI genes via the production of jasmonic acid (Farmer and Ryan, 1992). A detailed overview of signal molecules, and in particular, the role of systemin in signalling the rapid systemic accumulation of PIs, is provided by Enyedi *et al.,* (1992) and Schaller and Ryan (1995).

1.3.3 Biological function of serine proteinase inhibitors

1.3.3.1 Defence-related roles

Although the elucidation of the role of PIs is incomplete, the current view is that they are primarily involved in the protection of plants from pests and possibly pathogens. The observation that they specifically inactivate proteinases such as trypsin, chymotrypsin, elastase and subtilisin of animal and microbial origin (Ryan, 1966; Richardson, 1977; Hilder *et al.,* 1987; Christeller and Shaw, 1989; Lorito *et al.,* 1994), while rarely inhibiting endogenous plant enzymes (Ryan and Walker-Simmons, 1981), is circumstantial evidence for this conjecture. Furthermore, PIs accumulate to high levels in tomato, potato and tobacco leaves after wounding (Green and Ryan, 1972; Graham *et al.,* 1986; Pearce *et al.,* 1993). The finding that PIs are induced by pathogen attack

(Peng and Black, 1976; Gatehouse *et al.,* 1979; Roby *et al.,* 1987; Geoffroy *et al.,* 1990; Pautot *et al.,* 1991) also lends support to the hypothesis that PIs are involved in plant protection.

1.3.3.2 Other biological activities

In addition to the evidence that PIs are involved in plant defence, other functions are known. Proteinase inhibitors found in seeds and tubers may function as storage proteins (also see sections 1.2.1.1 and 1.4.3.3). The cysteine residues in PIs may provide a source of sulphur for germinating seeds. Other cysteine-rich proteins like the thionins (Bohlmann, 1994) and 2S albumins are thought to function in this way (Terras *et al.,* 1993a). An amino acid storage role for PIs is deduced from the fact that their activity and level decline during seed germination (Richardson, 1991). The sulphur from sulphur-containing amino acids may become mobilised as the PI is degraded together with the other stored reserves.

The process of seed maturation and germination requires endopeptidases to mobilise as well as cleave storage proteins (Hara-Nishimura *et al.,* 1991; Scott *et al.,* 1992; Abe *et al.,* 1993; Pueyo *et al.,* 1993). Proteinase inhibitors could be involved in the regulation of proteinase activity, although very few of the serine PIs characterised so far inhibit endogenous proteinases (Ryan and Walker-Simmons, 1981). There is, however, evidence that a metallo-proteinase inhibitor is responsible for modulating endogenous protease activity in buckwheat seeds, especially during germination (Dunaevskii *et al.,* 1995).

1.3.4 Serine proteinase inhibitors as putative defence genes in transgenic plants

1.3.4.1 Fungicidal activity

The considerable diversity of plant PIs and their role in defence is reinforced by early studies documenting the fungicidal activity of proteinase inhibitors (Senser *et al.,* 1974; Mosolov *et al.,* 1976; Mosolov *et al.,* 1979; Mosolov and Shul'gin, 1987). The induction of proteinase inhibitors in response to fungal elicitors (Roby *et al.,* 1987;

Rickauer *et al.,* 1989) or infection (Peng and Black, 1976; Roby *et al.,* 1987) also supports the view that these molecules may play a role in plant protection.

There are two current hypotheses to explain the inhibitory effects of PIs on fungi. Fungal pathogens secrete proteolytic enzymes which possibly play a role, either in fungal development and proliferation (Kuc and Williams, 1962; Porter, 1966) or as virulence factors (cited in Reddy *et al.,* 1996). It has been postulated that PIs might arrest fungal invasion by directly inhibiting this proteolytic activity (Brown and Adikaram, 1983; Ryan, 1990). Serine proteinase inhibitors potentially inhibit fungi which secrete serine proteases during invasion of their host. Examples of fungi with serine proteolytic activity include *Trichophyton rubrum* (Lambkin *et al.,* 1996), *Ophiostoma piceae* (Abrahams and Breuil, 1996), *Streptomyces* species (Bono *et al.,* 1996; Demina and Lysenko, 1996) and *Botrytis cinerea* (Brown and Adikaram, 1983). Cell wall proteins isolated from infected tomatoes were able to inhibit 70% of the activity of the proteases produced by *B. cinerea* (Brown and Adikaram, 1983), providing evidence that chymotrypsin and trypsin-inhibitors were induced and active at the site of infection. However, the cell wall proteins did not affect proteases from two other pathogenic fungi infecting tomato (Brown and Adikaram, 1983), indicating that there is likely to be a species-specific interaction between PI and the fungal pathogen.

A second hypothesis postulates that PIs inhibit spore germination and germ tube elongation of phytopathogenic fungi by causing cytoplasmic leakage (Lorito *et al.,* 1994). In this *in vitro* study, cabbage leaf PIs exhibited antifungal activity towards two plant pathogenic fungi which do not normally infect cabbage: *B. cinerea* and *Fusarium solani.* While the reasons for the observed antifungal activity remain unknown, the authors propose that the chymotrypsin and trypsin PIs from cabbage leaves inhibit the synthesis of chitin, which is one of the main cell wall components of fungal hyphae. As a result of containing less chitin, hyphae would have weaker cell walls which might result in the leakage of cell contents (Lorito *et al.,* 1994). A more thorough investigation is required to understand the indirect effects of PIs on chitin synthesis. Interestingly, even though the PIs were isolated from cabbage, they had no effect on a fungus that attacks

this plant (Lorito *et al.,* 1994). Likewise, a chymotrypsin inhibitor from potatoes does not inhibit the extracellular protease of *Erwinia carotovora* ssp. *carotovora* (a fungal potato pathogen) which provides further evidence of PIs may have specificity only for certain fungi (Heilbronn and Lyon, 1990).

So far, there are no reports that transgenic plants containing PIs are less susceptible to fungal pathogens. Because the *in vitro* evidence for antifungal activity of PIs is substantial, I examined whether transgenic tobacco plants containing a PI gene were protected against fungal infection.

1.3.4.2 Insecticidal activity

Because plant proteinase inhibitors rarely inhibit endogenous plant proteases, there have been a number of stages in demonstrating whether PIs inhibit insect proteases. The first entailed the identification and characterisation of midgut proteases in different insect families. Lepidopteran species primarily use serine endopeptidases for digestion, for example: trypsin, chymotrypsin, elastase and carboxypeptidase A and B (Johnston *et al.,* 1991; Christeller *et al.,* 1992; Xu and Qin, 1994), whereas major proteolytic activity in the midgut of Coleopteran species is from cysteine and aspartate proteases (Wolfson and Murdock, 1987; Michaud *et al.,* 1995; Edmonds *et al.,* 1996), with some exceptions (Christeller and Shaw, 1989; Bian *et al.,* 1996).

The potential for plant PIs to inhibit insect gut proteases has commonly been assessed by *in vitro* inhibition assays. The inhibitory effect of serine PIs on midgut protease activity varies between species, according to the classes of proteases they contain (Christeller *et al.,* 1992; Broadway and Villani, 1995). Of particular interest to this thesis is that potato inhibitors I and II (which have chymotrypsin and/or trypsin inhibitory characteristics) inhibited casein hydrolysis in crude gut extracts from *Helicoverpa armigera* by 90% and *H. punctigera* by 80% (Christeller, *et al.,* 1992). However, the inhibition of midgut protease activity *in vitro* does not always translate into an effect on insect physiology *in vivo*. This was the case for cabbage proteinase inhibitors which substantially inhibited the midgut protease activity of *Trichoplusia ni, Lymantria dispar* and *Helicoverpa zea in vitro,* yet when ingested, the PIs only retarded growth and development in *T. ni*

(Broadway, 1995). This report demonstrated, for the first time that some insects can change the relative proportion of their digestive enzymes in response to ingested PIs (Broadway, 1995).

The second stage of testing PIs on insects has been to include them in artificial diets. For example, when purified PIs from soybean and potatoes were incorporated into an artificial diet and fed to *H. zea* and *Spodoptera exigua* larvae, they substantially inhibited growth (Broadway and Duffy, 1986a). In other studies, soybean PIs in artificial diets reduced the mean growth rate of *Manduca sexta* (Shuckle and Murdock, 1983), *Ostrinna nubilalis* (Larocque and Houseman, 1990), *H. armigera* (Johnston *et al.*, 1993) and S*podoptera litura* (McManus and Burgess, 1995). The inclusion of PIs with known activity in artificial diets has also been used to provide information about the active array of midgut proteases. For example, only PIs with trypsin-and/or elastase-inhibitory activity were effective in reducing growth of black field crickets (*Teleogryllus commodus*), which indicated the predominant protease activity was likely to be from trypsin and elastases (Burgess *et al.*, 1991). Similarly, because antimetabolic effects were observed when *Callosobruchus maculatus* were fed diets containing cowpea trypsin inhibitor (Gatehouse and Boulter, 1983) and the cysteine proteinase inhibitor, E-64 (Murdock *et al.*, 1988), it was concluded that the midgut contains both serine and cysteine proteases.

In addition to producing new proteases in response to ingesting PIs, insects can also over produce proteases, leading to a deficiency of essential amino acids (particularly those rich in sulphur), which in turn, retards insect growth (Broadway and Duffey, 1986b; Ryan, 1990). The replacement of essential amino acids in the diet can nullify the effect of the PI. For example, in the case mentioned above, the observed growth inhibition when *C. maculatus* was fed cowpea trypsin inhibitor became insignificant when the artificial diet was supplemented with methionine and cysteine (Gatehouse and Boulter, 1983). Indeed, the effect of PIs included in artificial diets needs to be considered carefully as there is often an interaction between the amount of dietary protein and the PI. For example by doubling the protein content in the artificial diet, the inhibitory effect

of the trypsin and elastase inhibitors on *T. commodus* was reduced (Burgess *et al.*, 1991), perhaps by supplying a source of essential amino acids. In other reports, the inclusion of cysteine proteinase inhibitors in artificial diets was detrimental to *Callosobruchus chinenis, Riptortus clavatus* (Kudora *et al.*, 1996), *Hypera postica* (Elden, 1995) and *Diabrotica udecimpunctata howardi* (Edmonds *et al.*, 1996). The use of artificial diets to study the interaction of PIs has proved to be an effective tool in the evaluation of proteinase inhibitors as insect pest resistance factors in transgenic plants because it is possible to match particular PIs with target pests.

1.3.4.3 Using PI genes in transgenic plants to increase productivity

The third stage of assessing the insecticidal activity of serine PIs is to transfer the gene encoding the PI into transgenic plants and expose the plants to insect attack. The transfer of a trypsin inhibitor gene from cowpea (CpTI) into tobacco yielded plants that were more resistant to tobacco hornworm than the non-transgenic tobacco plants (Hilder *et al.*, 1987). CpTI was selected as a candidate for gene transfer because it was shown to be effective in artificial diets against a range of insect storage pests, including members of the Lepidoptera, Coleoptera and Orthoptera. An advantage of CpTI is that even at levels of up to 10% of the dietary protein, it was not toxic to mammalian cells (Pusztai *et al.*, 1992). When proteinase inhibitor II (pin2) from tomato, a powerful inhibitor of both trypsin and chymotrypsin, was transformed into tobacco, leaves from this plant retarded the growth of *Manduca sexta* (tobacco hornworm) (Johnson *et al.*, 1989). It appears that the ingestion of transgenic tobacco containing PIs can affect the growth of insect pests differentially. For example, *Chrysodeixis eriosoma* grew more slowly when fed leaves from tobacco plants transformed with pin2, yet *Spodoptera litura* or *Thysanoplusia orichalcea* larvae were unaffected (McManus *et al.*, 1994). While McManus *et al.*, (1994) demonstrated that a single PI shows differential toxicity to insects, it has also been observed that insects are only susceptible to certain PIs. For example, transgenic tobacco containing the Kunitz soybean trypsin inhibitor were completely resistant from attack by *S. litura* (Marchetti *et al.*, 1994) while pin2 was innocuous to these same insects (McManus *et al.*, 1994).

However, there are limitations to the efficacy of PIs in transgenic tobacco plants. Jongsma *et al.,* (1995) for example, recently reported that *Spodoptera exigua* larvae fed transgenic tobacco containing potato pin2 compensated for loss of trypsin activity by inducing new protease activity that was insensitive to inhibition by pin2. Broadway (1995; 1996) also reported that insects (e.g. *H. zea, Pieris rapae, M. sexta*) were able to alter the complement of midgut proteases in response to proteinase inhibitors and detailed the importance of matching multiple inhibitors to the complement of midgut enzymes in target pests in order to maximise the protective effects of PIs. Another possible way by which insects can become resistant is that an insect can modify the same protease so that the interaction between proteinase inhibitors and the target proteinase is no longer possible. Moreover, the use of tobacco as a host for transgenes must be evaluated carefully. After seven days, high levels of endogenous PIs can be induced in leaves as a result of wounding and the effects of these endogenous inhibitors may confound the effects of the introduced transgene (Jongsma *et al.,* 1994).

In addition to tobacco, serine PI genes have been transferred to cotton and rice. PIs derived from *Manduca sexta* were transferred into cotton plants and resulted in reduced reproduction of *Bemisia tabaci* (sweet potato whitefly) (Thomas *et al.,* 1995b). Two independent studies have demonstrated that rice transformed with either pin2 or CpTI were more protected from attack by *Sesamia inferens* (Duan, *et al.,* 1996; Xu *et al.,* 1996), which confirms the potential for serine PIs to provide protection to important crop species. Finally, there has been one report on the use of cysteine PIs in transgenic plants. Oryzacystatin from rice was transferred into poplars and trees were more tolerant to *Chrysomela tremulae* (Leplé *et al.,* 1995).

Table 1-4 provides a summary on the toxicological effects of PI genes on insect pests, after their transfer into plants using the examples discussed here.

Table 1-4. Insecticidal effects of transgenic plants containing PIs

SERINE PI	INSECT	TRANSGENIC PLANT AND EFFECT	REFERENCE
Cowpea Trypsin inhibitor (CpTI)	*Helicoverpa virescens*	Tobacco: enhanced resistance	Hilder *et al.*, 1987
CpTI	*Sesamia inferens*	Rice: significantly increased resistance	Xu *et al.*, 1996
	Chilo suppressalis		
Tomato pin1	*Manduca sexta*	Tobacco: very little effect	Johnson *et al.*, 1989
Tomato pin2 and Potato pin2	*Manduca sexta*	Tobacco: enhanced resistance	Johnson *et al.*, 1989
	Chrysodeixis eriosoma	Tobacco: slower development	McManus *et al.*, 1994
	Spodoptera litura	Tobacco: no effect	McManus *et al.*, 1994
	Spodoptera exigua	Tobacco: no effect - insects adapt to PIs	Jongsma *et al.*, 1995
	Sesamia inferens	Rice: increased resistance	Duan *et al.*, 1996
Kunitz Soybean Trypsin inhibitor	*Spodoptera litura*	Tobacco: complete resistance	Marchetti *et al.*, 1994
***Manduca sexta* PIs**	*Bemisia tabaci*	Tobacco: reduced reproduction rate	Thomas *et al.*, 1995a
		Cotton: reduced fecundity	Thomas *et al.*, 1995b
Multidomain PI from *Nicotiana. alata* (Na-PI)	*H. armigera* and *H. punctigera*	Tobacco: slightly enhanced resistance	This thesis and Charity *et al.*, 1997
	H. armigera and *H. punctigera*	Peas: slightly enhanced resistance	This thesis and Charity *et al.*, 1997
	Redlegged earth mite	Subterranean clover: Interaction unresolved	This thesis
CYSTEINE PI	**INSECT**	**TRANSGENIC PLANT AND EFFECT**	**REFERENCE**
Rice Oryzacystatin	*Chrysomela tremulae*	Poplar: toxic effects, increased tolerance	Leplé *et al.*, 1995
TWO DEFENCE GENES	**INSECT**	**TRANSGENIC PLANT AND EFFECT**	**REFERENCE**
CpTI and pea lectin	*H. virescens*	Tobacco: protective effects are additive	Boulter *et al.*, 1990
Na-PI and barley β-hordothionin	*H. armigera*	Tobacco: enhanced resistance with 2 genes	This thesis

1.3.5 Characterisation and processing of the proteinase inhibitor of *Nicotiana alata* (Na-PI)

The proteinase inhibitor gene from the stigmas of *Nicotiana alata* (Na-PI) is a novel gene for transfer into plants as it produces five homologous 6kDa PIs and two flanking regions from a single 40.3kDa precursor (Atkinson, 1992; Atkinson *et al.*, 1993a) (Fig. 1-1). Most other serine PIs are synthesised as low molecular weight primary translation products that undergo minimal modification and have only one or two reactive sites. Four of these Na-PIs exhibit inhibitory activity against trypsin and one inhibits chymotrypsin (Atkinson *et al.*, 1993a; Heath *et al.*, 1995). Each of the proteinase inhibitors are about 53 amino acids (Heath *et al.*, 1995) and contain eight cysteine residues which are stabilised by four disulphide linkages (Nielsen *et al.*, 1995). After cleavage of the precursor, it is proposed that the N-terminal and C-terminal regions form a putative sixth domain with a chymotrypsin reactive site (indicated by 'C', in Fig. 1-1) (Nielsen *et al.*, 1996).

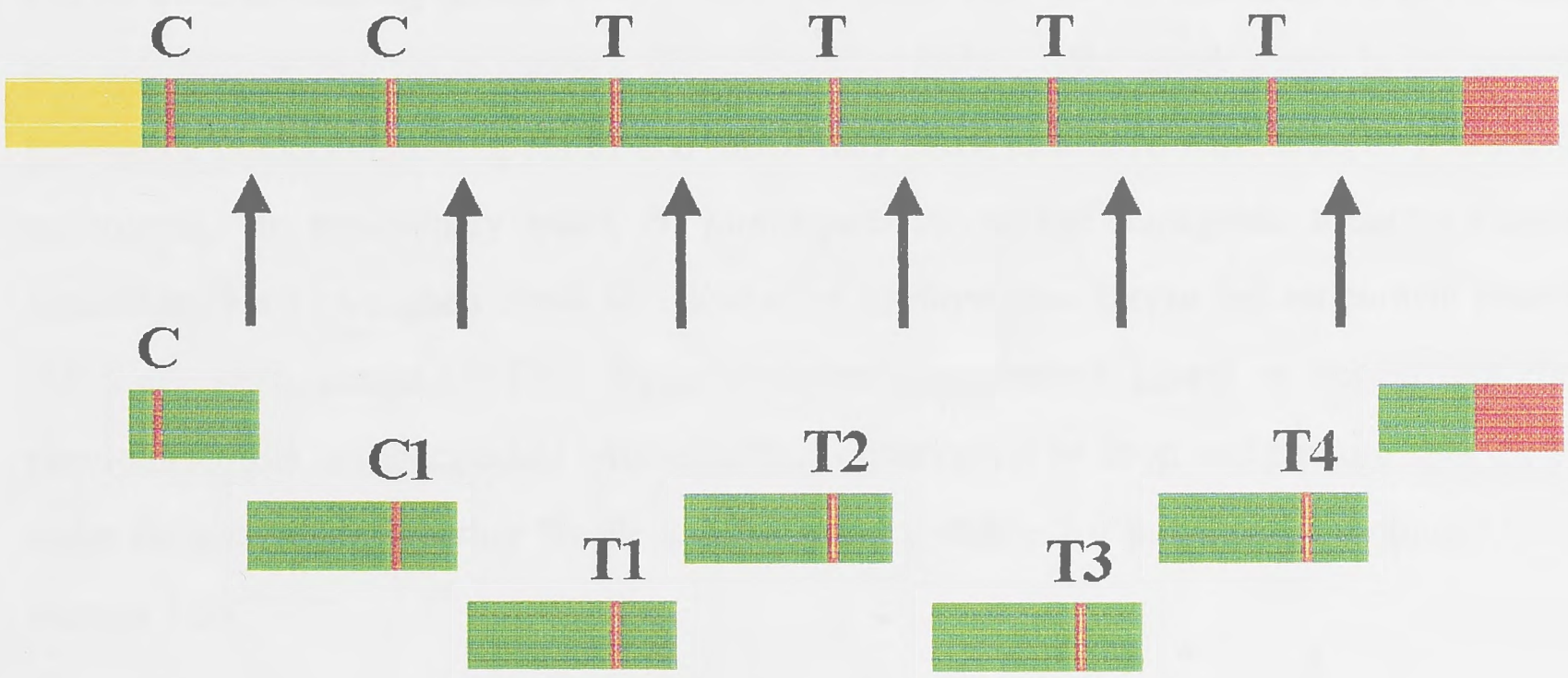


Fig. 1-1. The structure and processing of the *Nicotiana alata* Proteinase Inhibitor. The precursor has a signal sequence and six repeated domains which are proteolytically cleaved at the sites indicated by arrows. Cleavage produces five peptides: C1, chymotrypsin inhibitor and T1-T4, trypsin inhibitors and two flanking regions. The solid bars represent the reactive sites of the inhibitors which are marked 'C' for chymotrypsin reactive site or 'T' for trypsin reactive site. (Figure adapted from Heath *et al.*, 1995).

These polypeptides have sequence similarity to the type II serine PIs from tomato, potato and eggplant (Atkinson *et al.*, 1993a). The unprocessed precursor for Na-PI has anti-chymotrypsin and anti-trypsin activity but processing of the molecule enhances its trypsin

inhibitory activity, presumably by exposing all four trypsin inhibitory sites (Heath *et al.*, 1995).

1.3.6 Does Na-PI have a role in plant defence?

As described earlier for other serine PIs (section 1.3.4.2 and 1.3.4.3), three stages in the analysis of the interaction of PIs with insects, are also being used to assess the potential of Na-PI to confer insect resistance to crops. First, *in vitro* tests were used to determine the effect of the chymotrypsin inhibitor (C1) and the trypsin inhibitors (T1-T4) (from the cleaved or uncleaved Na-PI precursor) against the protease activity of gut extracts from a range of insects. The effect of Na-PIs on casein hydrolysis varied from 37-77% inhibition depending on the insect gut extract being tested (Heath *et al.*, 1997). Na-PIs inhibited gut proteases from five insect orders *in vitro* and displayed significant inhibitory activity against *Heliocoverpa armigera* (tobacco budworm), *H. punctigera* and the black field cricket (Heath, 1994; Heath *et al.*, 1997). Secondly, *H. punctigera* fed on diets containing 0.26% (w/w) Na-PI weighed about 54% less after 12 days, than the caterpillars fed on a control diet (Heath, 1994). The third stage is to create transgenic plants containing Na-PI and test if they show enhanced resistance to pests and pathogens. In preliminary trials, *H. punctigera* larvae fed transgenic tobacco leaves containing Na-PI weighed about 56% less after 14 days, than larvae fed on control plants (M. Lee; pers. comm.). This thesis describes experiments aimed at confirming the previous results in tobacco and extending this observation to crop and pasture species in order to investigate whether Na-PI is a suitable candidate for possible pest control (see section 1.5).

1.4 Thionins

Thionins are a family of small (M_r ~5000), proteins described mainly in cereals (maize, barley, wheat and rye) and more recently in potato and *Arabidopis* (Epple *et al.*, 1995). Their name is derived from the Greek word meaning 'sulphur' because they are rich in cysteine residues (Florack and Stiekma, 1994). They have been found in seeds, stems, roots. Thionins are basic proteins and exert toxicity to bacteria, fungi, yeasts and some eukaryotic cells *in vitro* by largely unknown mechanisms, though suggestions are

discussed in section 1.4.3.2. Thionins have been the subject of many reviews; García-Olmedo *et al.,* (1989); Bohlmann and Apel (1991); García-Olmedo *et al.,* (1992); Florack and Stiekma (1994) and Bohlmann, (1994).

1.4.1 Distribution and types of thionins

There are five types (I-V) of thionins based on their organ specificity, species of origin and their structural properties (García-Olmedo *et al.,* 1992). Four types of thionins show amino acid sequence similarity. In particular, the positions of the cysteine residues are highly conserved and their tertiary structure is stabilised by disulphide bridges (Florack and Stiekma, 1994). Type I thionins are abundant in cereal endosperms, have four disulphide bonds and are typically 45 amino acids in length. Well studied examples include the α- and β-hordothionins from barley endosperm, (Hernández-Lucas *et al.,* 1986; Ponz *et al.,* 1986) and α1-, α2- and β-purothionins from wheat (Mak and Jones, 1976; Castagnaro *et al.,* 1994). The seven sequences known to date are highly basic and have no negatively charged residues (García-Olmedo *et al.,* 1992). Immuno-gold electron microscopy has been used to show that the subcellular location of type I thionins in wheat and barley endosperm is electron-dense spheroides in the periphery of protein bodies (Carmona *et al.,* 1993a).

Type II thionins have been found in barley leaves (Bohlmann and Apel, 1987; Gausing, 1987) and in the nuts and leaves of *Pyrularia pubera* (Vernon *et al.,* 1985). They also contain four disulphide bonds but are less basic than type I thionins and are either 46 or 47 amino acids long.

Type III thionins have three disulphide bonds, 46 amino acids and are also less basic than type I thionins. A mixture of Type III thionins was first isolated nearly 50 years ago from European mistletoe species and named viscotoxins (Winterfeld and Bijl, 1948; cited in Bohlmann, 1994). Since that time, many viscotoxins have been characterised from mistletoe leaves and stems (Samuelsson and Pettersson, 1971; Olson and Samuelsson, 1972) and more recently from seeds (Schrader-Fisher and Apel, 1993). So far, there has been little molecular characterisation of type III thionins and none have been sequenced.

Type IV thionins are commonly called crambins because they were isolated from the seeds of the Abyssinian cabbage (*Crambe abyssinica*) (Teeter *et al.,* 1981; Vermeulen *et al.,* 1987). Crambins are not toxic (Van Etten *et al.,* 1969), they have 3 disulphide bonds, are 46 amino acids in length and are neutral. Type V (or γ-) are neutral (García-Olmedo *et al.,* 1992) and have recently been discovered in wheat (Colilla *et al.,* 1990; Castagnaro *et al.,* 1992) and barley endosperms (Mendez *et al.,* 1990). They do not share the same degree of structural similarity as the first four types. Two cysteine residues are missing, with consequent loss of the first and second disulphide bridges, which may allow a new disulphide bridge to be formed between the two mismatched cysteines (García-Olmedo *et al.,* 1992). As the γ-thionins are structurally unrelated to thionins, they have recently been reclassified as being members of a class of antimicrobial peptides called 'plant defensins' (Terras *et al.,* 1992b; Bohlmann, 1994) (section 1.2.1.1). A sixth grouping may be introduced to accommodate a new Ω-hordothionin, recently characterised from barley endosperm (Bruix *et al.,* 1995). This Ω-hordothionin has structural similarity to γ-thionins, scorpion toxins and insect defensins (Bruix *et al.,* 1995).

1.4.2 Structure, synthesis and processing of thionins

Comparison of thionin amino acid sequences indicates that the positions of the six or eight cysteines in thionin types I to IV are always conserved. The cysteine residues form disulphide-bridges, stabilising the molecule. In addition, tyrosine or phenylalanine at position 13 is conserved (except for type IV). Thionins are compact, amphipathic (one side hydrophilic, one side hydrophobic) molecules and are particularly amenable to structural studies as they crystallise readily with high yields (García-Olmedo *et al.,* 1989). The 3-dimensional structure of types I, III and IV is essentially the same (García-Olmedo *et al.,* 1992). They are 'L-shaped' with two α-helixes making up the long arm of the L, while the horizontal arm consists of two short antiparallel β-sheets (Bohlmann, 1994).

Type I thionins from barley endosperm are synthesised on membrane-bound polysomes as precursors requiring cleavage (Ponz *et al.,* 1983). The pre-proprotein has an N-terminal signal peptide, which is co-translationally cleaved, a sequence corresponding to the mature protein and an acidic peptide at the C-terminus (Ponz *et al.,* 1986) (Fig. 1.2). Type II and V have the same precursor structure (Gausing, 1987; Castagnaro *et al.,* 1992; respectively) and in all cases, the acidic peptide is cleaved off post-translationally (Ponz *et al.,* 1983). The function of the acidic peptide is not clear, but Bohlmann (1994) proposes it might neutralise the basic thionin domain, protecting host cells from its possible toxic effects. Alternatively, the acidic peptide might facilitate transport of the mature thionin through membranes (Florack *et al.,* 1994).

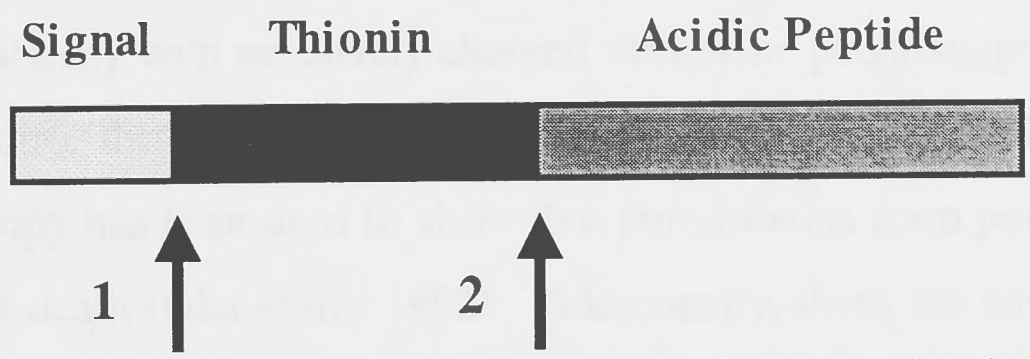


Fig. 1-2 Structure of the thionin precursor protein. All thionins have a signal sequence which must be co-translationally cleaved (indicated by the arrow labelled '1') and an acidic peptide which must be removed post-translationally (arrow '2'). (Figure modified from Ponz *et al.,* 1986).

1.4.3 Biological activity and function of thionins

1.4.3.1 Antimicrobial properties of thionins

The first report of the biological activity of thionins may have been the observation, that a substance in wheat flour was toxic to yeast (Jago and Jago, 1885; cited in Bohlmann, 1994). Over 50 years later, purothionins from wheat were characterised, purified (Balls and Hale, 1940) and their antimicrobial properties were documented (Balls *et al.,* 1942; Stuart and Harris, 1942). Since then, the toxicity of type I and II thionins to yeast (Okada *et al.,* 1970; Hernández-Lucas *et al.,* 1974), fungi (Bohlmann *et al.,* 1988) and bacteria (Fernandez de Caleya *et al.,* 1972) *in vitro* has been confirmed. Wheat β-purothionin displayed antifungal activity towards 12 phytopathogenic fungi (Cammue *et al.,* 1992) and barley leaf thionins (Type II) inhibited the growth of *Erisyphe graminis* f.sp. *hordei* (Bohlmann *et al.,* 1988). Purothionins from wheat (α_1, α_2, β) are also toxic to cultured spruce budworm and mosquito cells with LC_{50} values around 20 μg/ml (Jones

et al., 1985). These type II thionins also inhibited callus formation of plant protoplasts and the growth of the sugar cane fungus *Theielaviopsis pardoxa* (Reimann-Philipp *et al.,* 1989). Finally, there is one report that purothionins (and their counterparts from barley and rye) caused a 50% increase in insect mortality when injected into *Manduca sexta* larval hemocoel (Kramer *et al.,* 1979).

1.4.3.2 Effect of thionins on biological membranes

The precise mechanism of action of thionins is unknown, but the observation that they cause biological membranes to leak (Carrasco *et al.,* 1981; García-Olmedo *et al.,* 1983; Terras *et al.,* 1993a; Florack and Stiekma, 1994) has led to at least two hypotheses to explain their toxic effects. In one model, the positive residues of thionins are thought to interact electrostatically with negatively charged membrane phospholipids, causing pores or channels to form, through which the cell contents are released (Bohlmann, 1994). Electron microscopy has been used to show that purothionins form pores in membranes causing rapid cell death (Oka *et al.,* 1992). Additionally, there are similarities between the amino acid sequences of purothionins and the mammalian pore-forming protein EGF, particularly around the cysteine-rich motif (Oka *et al.,* 1992), although this evidence must be viewed with caution as the similarity is weak and does not include the first two cysteine residues (Bohlmann, 1994).

There is also evidence to support another mechanism in which it is proposed that thionins activate phospholipase by first binding to a membrane receptor (Vernon and Rogers, 1992). In this model, it is the detergent-like activity of the phospholipase and not the thionin itself which would cause membrane damage. *Pyrularia* thionin, for example, has been shown to bind to a specific phospholipid (Vernon and Rogers, 1992), leading to the stimulation of an internal phospholipase (Evans *et al.,* 1989) which permeabilises the membrane. One common feature is that calcium (5mM or greater) and other divalent metal ions reverse the haemolytic activity of thionins (e.g., Okada and Yoshizumi, 1973) - a well-documented characteristic of small, amphipathic, cysteine-rich antimicrobial molecules.

1.4.3.3 Other functions of thionins

Apart from suggestions that thionins have a role in protecting plants against microbial attack (Bohlmann and Apel, 1987; García-Olmedo *et al.,* 1989), thionins are implicated in other biological functions. Purothionins are able to reduce disulphide bonds on proteins (Wada and Buchanan, 1981) and activate phosphoinositide-mediated signal transduction (Angerhofer *et al.,* 1990). Together, these observations led to the proposition that thionins may function as secondary thiol messengers. Their reducing activity may explain why thionins inhibit ribonucleotide reductase (Johnson *et al.,* 1987), papainase (Balls *et al.,* 1942) and α-amylase (Jones and Meredith, 1982), however their subcellular location does not lend support to such cytosolic activity, nor is it clear how S-S reduction would occur, given that thionins have no free cysteine groups.

Jones and Meredith (1982) claimed that purothionins inhibited α-amylase activity. The significance of this observation is questionable because the amount of purothionin vastly exceeded the amount of α-amylase. Purothionins have also been shown to inactivate β-glucuronidase (GUS) and neomycin phosphotransferase (NPT-II) by attachment of the thionin to the enzyme through the formation of disulphide links (Piñeiro *et al.,* 1995). The formation of disulphide linkages was prevented by the addition of DTT (a strong reducing agent) as well as by preincubation of the enzyme in a sulphydryl blocking agent. The authors concluded that the reaction is selective as thionins did not react with all cysteine-containing proteins in a similar manner. Furthermore, when thionins were confronted with a complex protein mixture they only bound to one periplasmic component of the bacteria *Pseudomonas solanacearum* (Piñeiro *et al.,* 1995). One explanation is that this interaction could be related to the mechanism of toxicity, or as the *P. solanacearum* strain used was resistant to the thionin, perhaps to a resistance factor. Other studies on the purothionins have shown that they inhibit eukaryotic cell-free protein synthesis probably by directly binding to mRNA (García-Olmedo *et al.,* 1983).

Because type I thionins are abundant in endosperms and are sulphur-rich, these thionins may function as storage proteins, although it remains to be proven if sulphur originating from thionins is mobilised during seed germination. Nonetheless, there is some evidence

that sulphur from viscotoxins (type III) is mobilised during leaf maturation (Schrader and Apel, 1993). After detecting thionins in protein bodies, Carmona *et al.,* (1993a) suggested that they may be involved in packaging storage proteins into protein bodies.

1.4.3.4 Thionins and crop protection

The possibility that thionin genes could be appropriate candidates for conferring pathogen resistance on important crop species is based mainly on the following observations: (1) barley leaf thionins were induced after infection with powdery mildew (*Erysiphe graminis* f.sp. *hordei*) (Bohlmann *et al.,* 1994); (2) barley leaf thionins inhibit the growth of *Thielaviopsis paradoxa* and *Pyrenophora (drechslera) teres* in a plate diffusion bioassay (Bohlmann *et al.,* 1988); (3) β-purothionin is toxic to several phytopathogenic bacteria (Cammue *et al.,* 1992) and (4) the antifungal activity of thionins from wheat and barley *in vitro* could be enhanced when combined with 2S albumins (from oilseed rape) or barley trypsin inhibitors (Terras *et al.,* 1993a).

Initial studies with transgenic tobacco showed that barley α-hordothionin was expressed and cleaved in leaves. Furthermore, α-hordothionin isolated from leaves of these transgenic tobacco plants inhibited the plant pathogen *Clavibacter michiganensis* ssp. *michiganensis* in a similar to that of α-hordothionin purified from barley endosperm (Florack *et al.,* 1994) (Table 1-1). Subsequently, Carmona *et al.*, (1993b) found that the expression of a gene encoding α-hordothionin from barley endosperm in tobacco enhanced resistance to *Pseudomonas* species although the α-hordothionin homologue from wheat (α_1-purothionin) was not effective against these same bacterial pathogens (Carmona *et al.,* 1993b) (Table 1-1).

1.4.4 β-hordothionin from barley endosperm

There are representatives from type I (Ponz *et al.,* 1983), II (Bohlmann and Apel, 1987; Gausing, 1987) and V (Mendez *et al.,* 1990) thionins in barley. I describe experiments with type I β-hordothionin from barley endosperm. A cDNA clone (pTH2) encoding the precursor has been sequenced and characterised (Hernández-Lucas *et al.,* 1986). The precursor was composed of a 45 amino acid thionin sequence flanked by an 18 amino

acid signal peptide and a 64 amino acid acidic peptide which are removed co- and post-translationally, respectively (Ponz *et al.,* 1986). Complete chimeric α- and β-hordothionin genes have previously been transferred to tobacco (Florack *et al.,* 1994). The authors detail the processing, accumulation and antifungal activity of the α-hordothionin construct, but not the β-hordothionin construct. Much lower levels of α-hordothionin accumulated in transgenic tobacco made by Carmona *et al.,* (1993b) compared to those achieved by Florack *et al.,* (1994), yet expression was high enough to confer partial resistance to *Pseudomonas syringae* (Table 1-1). To date, as there are no reports of the effectiveness of transgenic tobacco containing β-hordothionin (β-HTH) on bacterial or fungal pathogens, I have sought to evaluate the potential of β-HTH for improving crop productivity.

1.5 Aims and scope of the present study

A review of the literature on the potential of antimicrobial and insecticidal genes to improve crop productivity, indicated that an increase in the pool of defence molecules amenable to genetic manipulation was highly desirable. My overall aim was to assess the potential of two defence genes to increase resistance of plants to pests and pathogens. As there is accumulating evidence to support the claim that proteinase inhibitors (PIs) and thionins are involved in the protection of plants, from pests and diseases I produced transgenic plants which contained the chimeric genes encoding a PI and a thionin. To describe the characterisation and investigation of the resistance of transgenic plants, this thesis is organised into five chapters:

Chapter One: Provides a review of the literature on possible defence genes with focus on proteinase inhibitors and thionins, drawing on relevant studies.

Chapter Two: A cDNA clone for a proteinase inhibitor had previously been isolated from the stigmas of *Nicotiana alata* (Na-PI) (Atkinson *et al.,* 1993a) and reconstructed as a chimeric gene for green tissue specific expression (A. Moore; pers. comm.). This chapter describes the transfer of this Na-PI chimeric gene firstly into tobacco, and then into two important grain and forage legumes, peas and subterranean clover, respectively.

The expression of Na-PI mRNA is analysed by northern blot analysis while western blotting provides information on the cleavage of the Na-PI precursor as well as quantifying Na-PI peptide accumulation. In addition, the stability of Na-PI in the germline of all three plant species is determined by observing the segregation ratio of the *na-pi* gene, for at least two generations. The second potential defence gene to be studied in this thesis is a β-hordothionin (β-HTH) from barley endosperm and this chapter also details the transfer and expression of the cDNA encoding this gene in tobacco. Finally, transgenic tobacco containing the highest amount of Na-PI and β-HTH were cross-fertilised to produce individual plants containing both genes. Evidence for F_3 double transformants being homozygous for both genes is provided by northern analysis.

Chapter Three: This chapter describes bioassays whereby the mortality, growth and development of insect pests is monitored, in order to test the performance of transgenic tobacco containing Na-PI in their leaves. In particular, tobacco and peas are assessed by feeding leaves from transgenic plants to *Heliocoverpa armigera* (tobacco budworm) and *H. punctigera* (native budworm), which are major pests of peas, cotton and other vegetable crops. Likewise, the insecticidal activity of subclover containing Na-PI on redlegged earth mite is investigated. Additionally, as a thionin gene may be a novel strategy to confer protection on tobacco against insects, the resistance of transgenic tobacco containing β-HTH is described using *H. armigera* as a test insect pest. In the same experiment, I test the proposal that pyramiding PIs and thionin genes offers greater protection to plants than either gene alone, so results from a bioassay testing if transgenic tobacco with two genes have improved resistance to *H. armigera* are presented.

Chapter Four: As the efficacy of PIs in transgenic plants against fungal pathogens has not been reported, tobacco plants containing Na-PI are examined for increased resistance to *Botrytis cinerea* (grey mould). Initial experiments use a bioassay on transgenic leaf pieces, while whole plants are used in later experiments This chapter also reports on the performance of transgenic tobacco containing β-HTH against *Pseudomonas solanacearum* (bacterial wilt) and *B. cinerea.* For reasons described above, the

protective effects of Na-PI and β-HTH in combination, on transgenic tobacco is assessed in bioassays with *B. cinerea* and *P. solanacearum.*

Chapter Five: This concluding chapter summarises both the major and minor findings of the experiments in this thesis and discusses the significance of the research in relation to previous studies. Finally, this chapter dwells on the implications of these conclusions and proposes strategies to further investigate the exploitation of defence genes to improve crop productivity.

Chapter Two

Plant Transformation

Chapter Two

Transformation and expression of genes for a proteinase inhibitor and thionin gene in transgenic plants.

2.1 Introduction

One of the most significant breakthroughs in plant science in recent years is the development of transformation systems which allow the transfer of genes from unrelated sources into crop plants. Genetic engineering may provide a strategy for improving the productivity of plants by introducing new genes that have the potential to increase pest and disease resistance. The overall aim of this thesis was to assess the potential of a proteinase inhibitor from *Nicotiana alata* and a β-hordothionin from barley, to increase the resistance of plants to pests and pathogens. The first requirement in realising this aim is the successful transformation and expression of these novel genes in plants. This chapter describes the transformation of tobacco, pea and subterranean clover with a proteinase inhibitor gene and tobacco with a thionin gene.

The precursor for a proteinase inhibitor found in the stigmas of *Nicotiana alata* (Na-PI) consists of six repeated domains that are cleaved to produce five homologous 6 kDa PIs (Fig. 1-1) (Atkinson *et al.*, 1993a). The presence of multiple inhibitory sites offers an advantage over single or double domain inhibitors in that five PIs will be produced from a single transcript, rather than the usual one gene: one protein ratio. This also means that the expression of these PIs would be co-ordinately regulated and may lead to accumulation of the inhibitors in transgenic plants. The precursor for Na-PI has chymotrypsin inhibitory activity but post-translational processing is required to expose all four trypsin inhibitory sites (Heath *et al.*, 1995). This means to maximise the biological usefulness of Na-PI as a defence molecule, the precursor must be cleaved in the leaves of

transgenic plants. The molecular analysis of plants transformed with Na-PI, therefore, is fundamental to the overall aim of the project.

In addition to Na-PI, this chapter describes the characterisation of transgenic tobacco containing β-hordothionin (β-HTH) from barley endosperm. Like Na-PI, the precursor for β-HTH requires cleavage. The pre-protein contains 127 amino acids including a signal sequence (18 amino acids), a mature thionin domain (45 amino acids) and an acidic domain at the C-terminus (64 amino acids). Active β-HTH is released after co-translational cleavage of the signal peptide and post-translational removal of the acidic peptide. Therefore, the success of β-HTH in providing plants with additional protection from pests and pathogens, depends primarily upon the cleavage of the β-HTH pre-protein in transgenic plants.

For initial studies, tobacco (*Nicotiana tabacum*), which is easily transformable (Horsch *et al.*, 1985) was chosen as a model system. Transformed plants were assessed for expression of the Na-PI and β-HTH gene constructs by northern and western blotting. Upon confirmation of cleavage of Na-PI and β-HTH in the leaves of transgenic tobacco, I wanted to extend these observations to important legume species, in order to assess if Na-PI will be processed and active in genera other than *Nicotiana,* and plant families other than the Solanaceae.

To this end, pea (*Pisum sativum* L.) and subterranean clover (*Trifolium subterranean* L.), were chosen as examples of important grain and forage legumes. Pea is an important food crop which provides a source of protein for both humans and animals. I utilised the pea transformation and regeneration system developed by Schroeder, H. *et al.,* (1993) to transfer the cDNA encoding Na-PI into the cultivar 'Greenfeast'.

Subterranean clover, hereafter referred to as subclover, is the most widely used pasture legume in temperate Australia. It is well adapted for grazing and is suitable for growth in neutral to slightly acidic soils (Gillespie, 1993). Given its ability to fix nitrogen, growth habit, nutritional value and palatability, it has established itself as a major contributor to

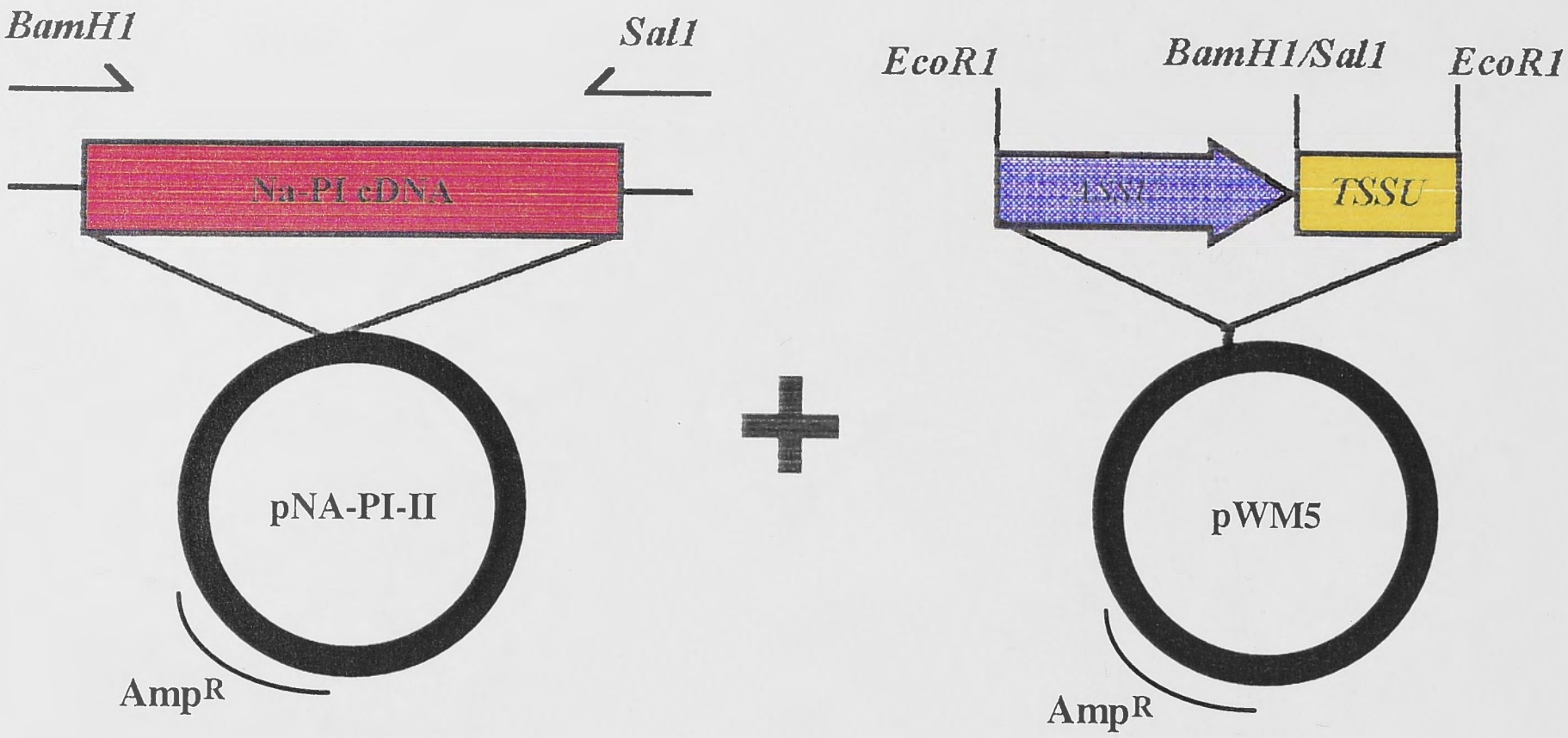
many primary industries in Australia especially those involved in meat, wool, dairy and wheat production (Johnstone and McLean, 1987). A rapid and reproducible transformation system developed for subclover (Khan *et al.,* 1994) allowed the introduction of the *na-pi* gene into this forage legume.

A briefer account of the transformation and expression of *na-pi* in tobacco and pea has been prepared for publication (Charity *et al.*, 1997).

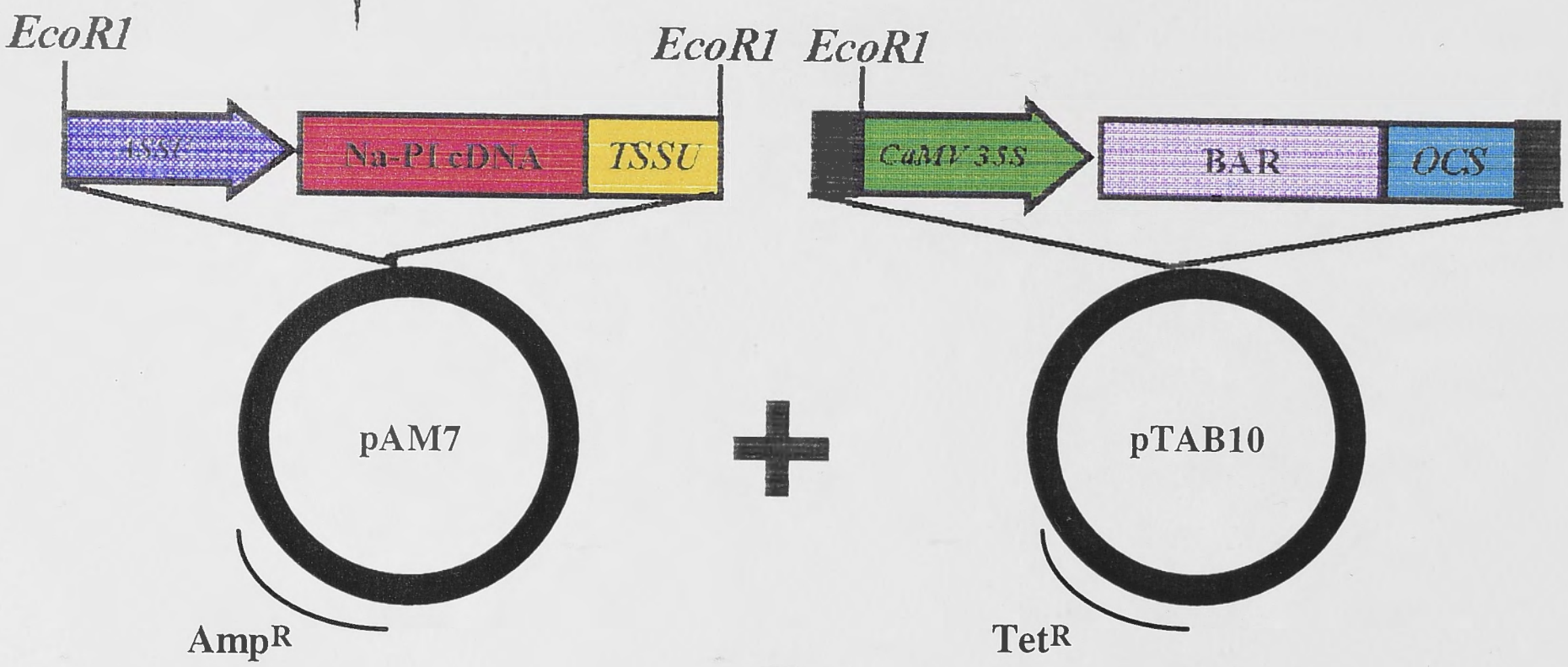
2.2 Material and Methods

2.2.1 Construction of pAM8 which contains the Na-PI cDNA

The Na-PI coding region (1.2 kb) was amplified from the plasmid pNA-PI-II (Atkinson *et al.,* 1993a) by PCR. The 5' amplification primer contained a *Bam*H1 site and the 3'amplification primer contained a *Sal*1 site for insertion of the PCR product into the plasmid, pWM5 (Tabe *et al.,* 1995). pWM5 contains the promoter and 5' untranslated region (UTR) from a gene from *Arabidopis thaliana* encoding the small subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase, (Rubisco) (Krebbers *et al.,* 1988b). This promoter, hereafter known as ASSU, is adjacent to a polycloning site, followed by a 3' UTR from the tobacco gene for the small subunit of Rubisco (TSSU) (Tabe *et al.,* 1995). The resulting plasmid (pAM7) was cut with *Eco*RI to release the complete chimeric gene. This 3.2 kb fragment was inserted into the *Eco*RI site between the T-DNA borders of the binary vector pTAB10 (Tabe *et al.,* 1995). pTAB10 also contains the selectable marker gene *bar* driven by the Cauliflower Mosaic Virus (CaMV) 35S promoter. This gene codes for phosphinothricin acetyl transferase which confers resistance to phosphinothricin (PPT), the active ingredient in the non-selective herbicide, glufosinate ammonium. The resulting binary plasmid (pAM8; Fig. 2-1) was mobilised from *Escherichia coli* into *Agrobacterium tumefaciens* strain AGL1, by triparental mating, using the helper plasmid pRK2013 (Ditta *et al.,* 1980). pAM8 was used to transform tobacco, pea and subclover. The plasmid pAM7 was constructed by Andrew Moore (CSIRO, Division of Plant Industry).



- PCR amplified to create *BamH1/Sal1* sites
- Digest PCR product with *BamH1 and Sal1*
- Digest pWM5 with *BamH1 and Sal1*



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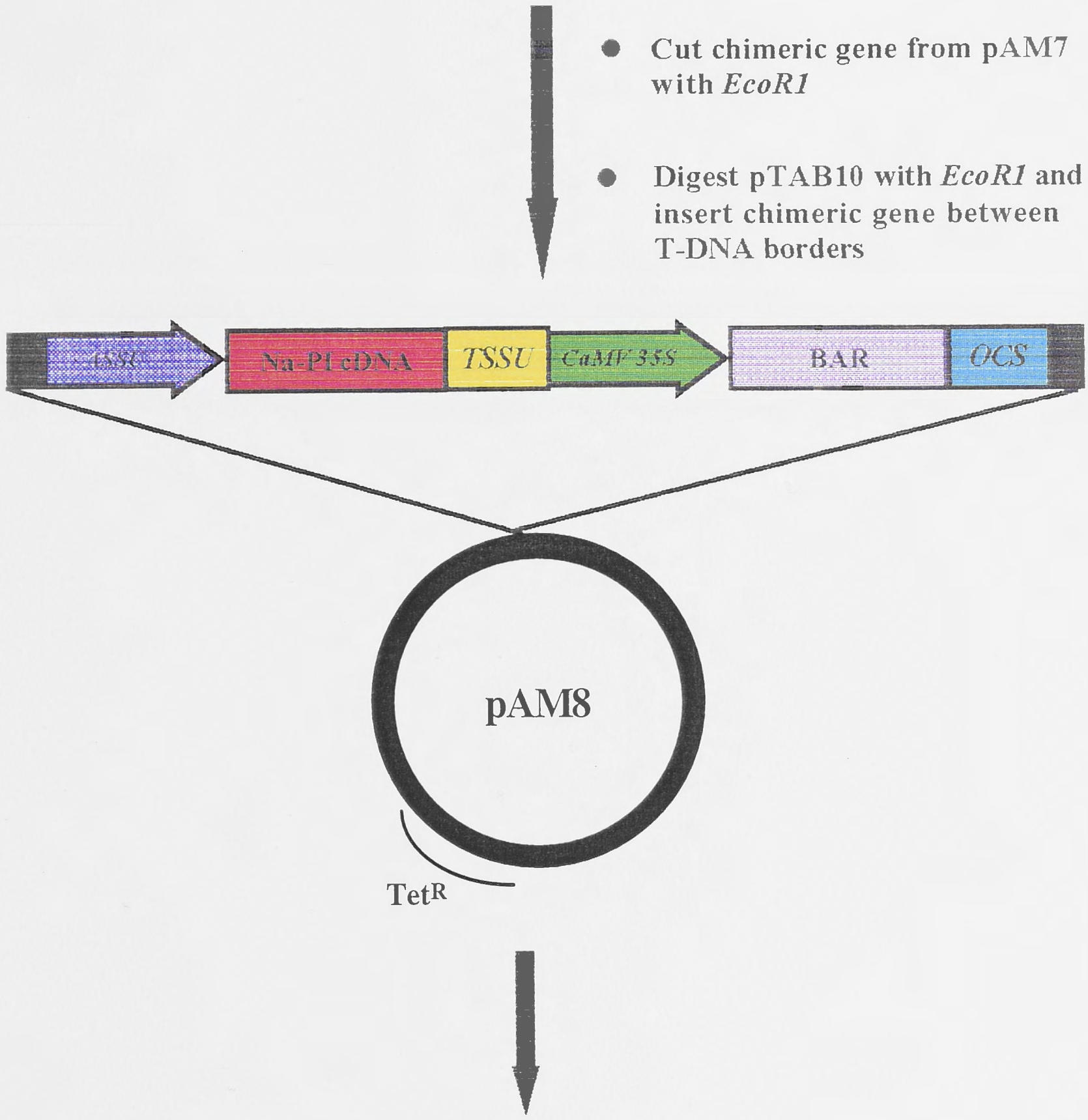
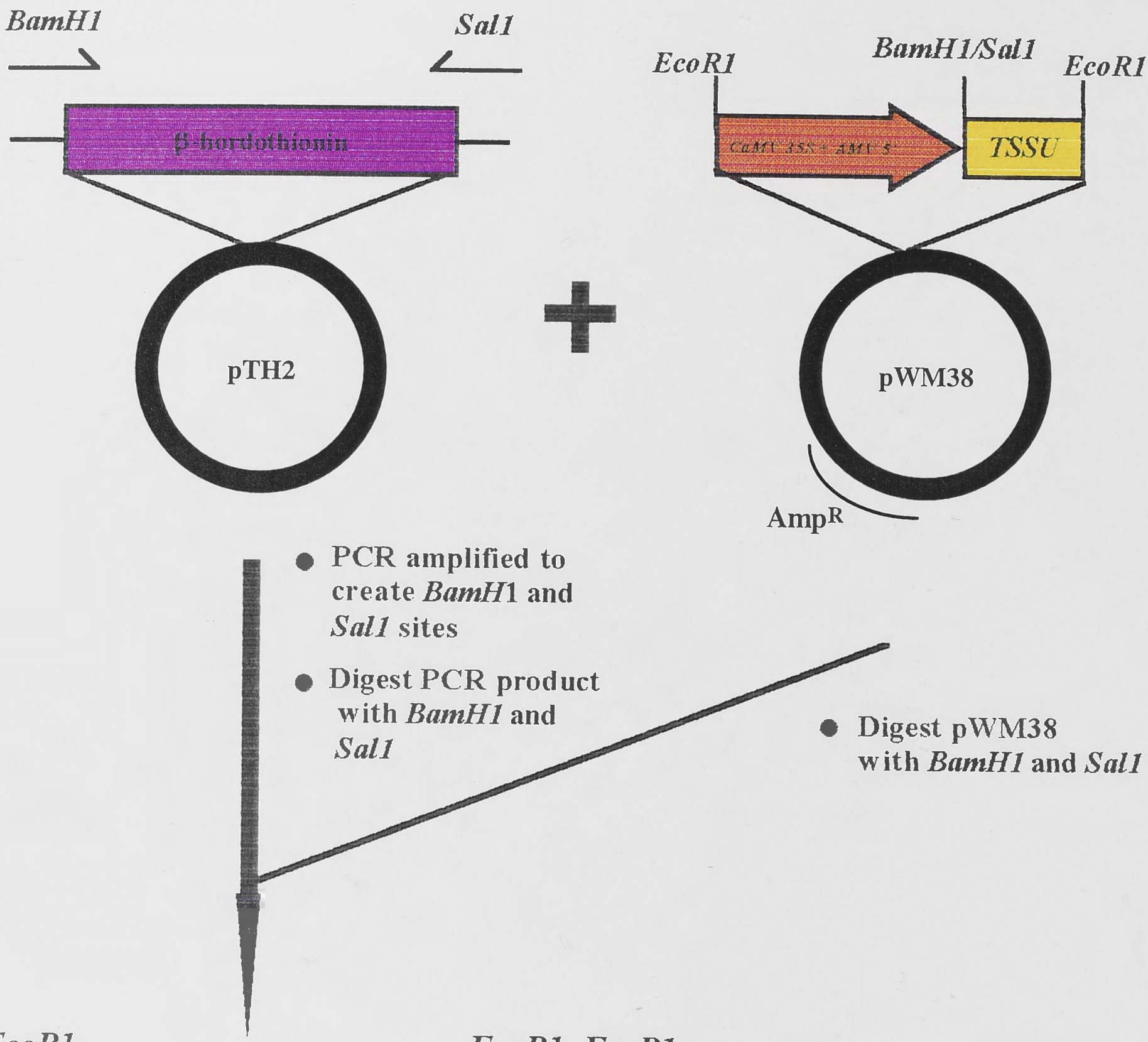


Agrobacterium tumefaciens (AGL1) & plant transformation

Fig. 2-1 Construction of the binary plasmid pAM8 containing a chimeric Na-PI gene.
Black open circles are schematic representations of plasmids, arrowed boxes are promoters and coloured boxes represent the cDNA as labelled. The plasmid pNA-PI-II contains the cDNA encoding the proteinase inhibitor from *N. alata* (Na-PI). *Bam*H1 and *Sal*1 sites were created at the 5' and 3' ends of the Na-PI cDNA, respectively, by PCR. The fragment was cut with *Bam*H1 and *Sal*1 and ligated between the *Arabidopsis* Rubisco small subunit promoter (ASSU) and the tobacco 3' UTR terminator (TSSU) of pWM5. The entire chimeric gene was released from pAM7 by *Eco*R1 digestion and inserted into the *Eco*R1 cut binary vector, pTAB10 to create pAM8. pAM8 is described fully in the text.


BamH1
Sal1
β-hordothionin
pTH2
EcoR1
BamH1/Sal1
EcoR1
CaMV 35S + AMV 5'
TSSU
pWM38
AmpR
PCR amplified to create BamH1 and Sal1 sites
Digest PCR product with BamH1 and Sal1
Digest pWM38 with BamH1 and Sal1
EcoR1
EcoR1
EcoR1
35S + AMV
β-HTH
TSSU
CaMV 35S
BAR
OCS
pWM39
pTAB10
AmpR
TetR

to next page

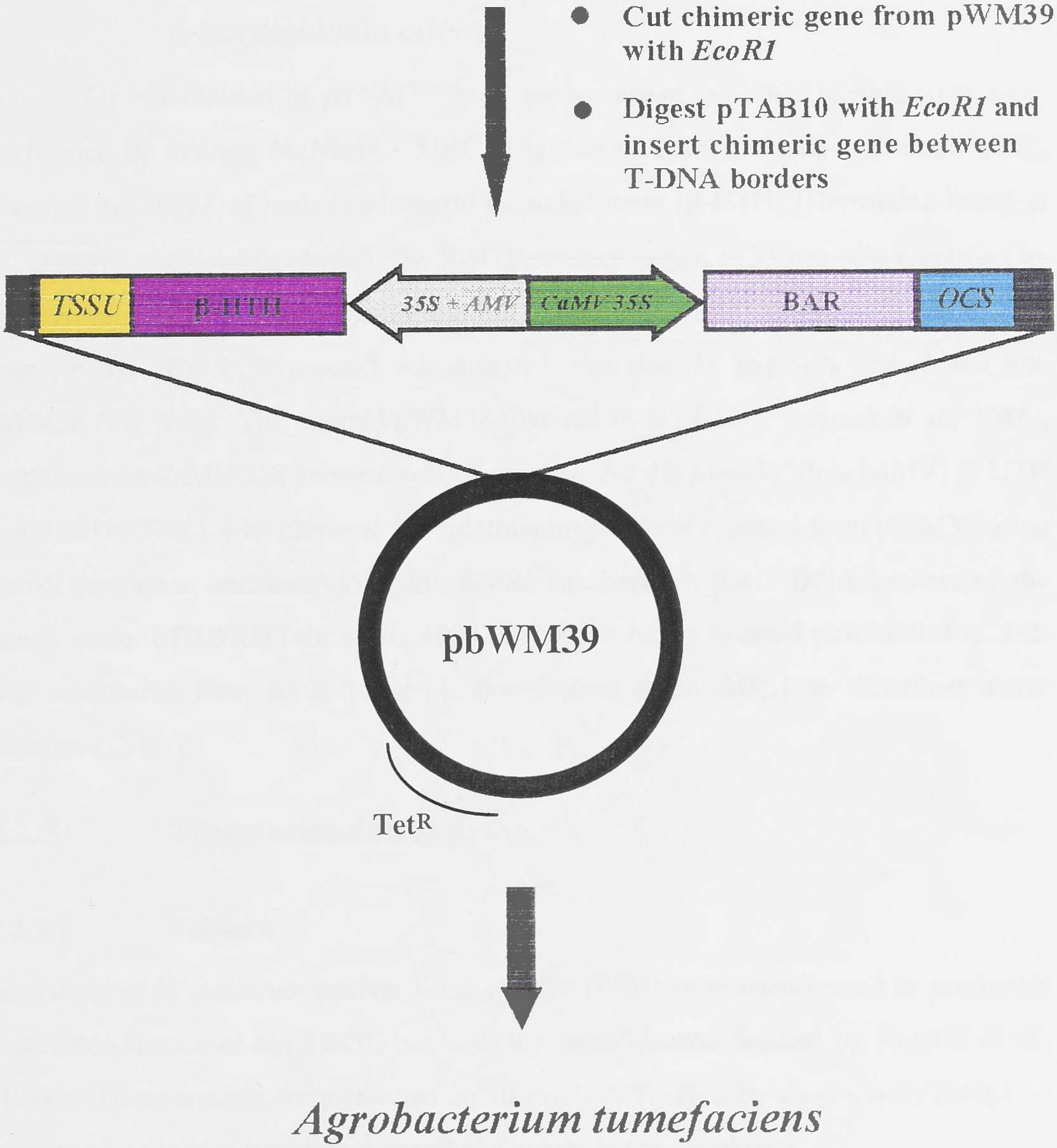


Fig. 2-2 Construction of the binary plasmid pbWM39 containing a chimeric β-hordothionin gene
PCR was used to construct *BamH1* and *Sal1* sites at the 5' and 3' ends, respectively of the β-hordothionin (β-HTH) cDNA of pTH2. The fragment was digested with restriction enzymes, *Bam*H1 and *Sal*1 and ligated into pWM38 at a *Bam*H1 site after the Cauliflower Mosaic Virus 35S (35S CaMV) promoter, creating a new plasmid, pWM39. pWM39 was cut with *Eco*R1, and the chimeric gene containing β-HTH was ligated into pTAB10 which had been digested with *Eco*R1. The binary vector was named pbWM39 and is described in detail in the text.

2.2.2 Construction of pbWM39 which contains the β-hordothionin cDNA

Except for mobilisation of pbWM39 into *Agrobacterium*, all other cloning steps were performed by Warren McNabb, CSIRO, Division of Plant Industry. Plasmid pTH2 contains the cDNA of barley endosperm β-hordothionin (β-HTH) (Hernández-Lucas *et al.,* 1986). To simplify cloning, the β-HTH coding region (420 bp) was amplified by PCR from pTH2 to create *Bam*H1 and *Sal*1 consensus sequences at the 5' or 3' end, respectively. The PCR product was digested with *Bam*H1 and *Sal*1 and cloned into pWM38 (Fig. 2-2). The plasmid pWM38 (derived from pDH51; Pietrzak *et al.,* 1986), contained the CaMV 35S gene expression cassette, Alfalfa Mosaic Virus (AMV) 5' UTR and TSSU. The 1.4 kb chimeric β-hordothionin gene was released from pWM39 using *Eco*RI restriction and inserted at the *Eco*RI site between the T-DNA borders of the binary vector pTAB10 (Tabe *et al.,* 1995). The new binary plasmid pbWM39 (Fig. 2-2) was transferred from *E. coli* into *A. tumefaciens* strain AGL1 as described above (section 2.2.1).

2.2.3 Transformation systems

2.2.3.1 Tobacco

Leaf disks of *N. tabacum* cultivar Wisconsin 38 (W38) were transformed as previously described (Horsch *et al.*, 1985), but with the modifications detailed by Higgins *et al.,* (1988). Transformants were selected on 10 mg/L PPT. Healthy shoots were rooted on hormone-free media, transferred to soil and grown in the glasshouse.

2.2.3.2 Pea

The *Agrobacterium*-mediated gene delivery system developed by Schroeder H. *et al.,* (1993) was used to transform the pea cultivar Greenfeast. Briefly, longitudinal segments of the embryonic axis from immature seeds, were co-cultivated with *Agrobacterium* containing pAM8 for three days. Following co-cultivation, callus was induced on media containing PPT (12.5 mg/L) for 15 days after which the calli were transferred to medium containing hormones and PPT (12.5 mg/L) to induce shoot formation. Shoots were

excised from callus and transferred to root-inducing medium containing PPT (10 mg/L). When adequate root growth was established, plantlets were transferred to soil in the glasshouse.

2.2.3.3 Subclover

The cultivar Trikkala was transformed by an *Agrobacterium*-mediated gene delivery system described by Khan *et al.,* 1994. Hypocotyl segments excised from imbibed seeds were co-cultivated for seven days with *Agrobacterium* containing pAM8. Explants were washed in cefotaxime (500 mg/L) to prevent further bacterial contamination and the growing root tip was discarded. The remaining 1-2 mm hypocotyl segment was placed on regeneration medium containing 50 mg/L PPT. Surviving explants were transferred to fresh medium every three weeks. Green shoots which emerged from the hypocotyl segments were excised and dipped in indole-3-butyric acid (IBA) (1 mg/ml) for one min to induce root formation. Shoots were grown in media supplemented with 3 mg/L IBA and plantlets with adequate root growth were transplanted into a well draining soil in the glasshouse (Khan *et al.,* 1994).

2.2.4 Northern blot analysis of transgenic plants

2.2.4.1 RNA extraction from leaves

Total RNA was isolated and purified from tobacco and pea tissues using a lithium chloride precipitation method (Chandler *et al.,* 1983). 300 mg of frozen young tissue was ground to a fine powder before extraction with 900 µl of buffer and 900 µl of phenol-chloroform-isoamyl alcohol (25:24:1). The nucleic acids were precipitated with an equal volume of isopropanol and the pelleted nucleic acids redissolved in water before reprecipitation with one volume of 4M lithium chloride. The absorbance at 260 nm was used to determine the concentration of RNA.

2.2.4.2 Gel electrophoresis and RNA blotting

Five micrograms of total RNA was separated by gel electrophoresis in a 1.4% agarose gel containing 0.66M formaldehyde and transferred to nylon membrane (Hybond^{TM}-N, Amersham) by capillary blotting in 20X SSC (1X SSC contains 0.15M sodium chloride

and 0.015M sodium citrate). DNA was ^{32}P-labelled to a high specific activity (10^8 dpm/mg) using a Megaprime DNA labelling system (Amersham). Probes were either the 1.2 kb Na-PI cDNA coding region from pAM8, the 450 bp β-HTH fragment from pbWM39 or DNA encoding the wheat 18S ribosomal RNA (Gerlach and Bedbrook, 1979). The 18S ribosomal probe accounted for differences in loading, enabling the quantification of the Na-PI mRNA by ImageQuant analysis (Molecular Dynamics, Version 3.3). Hybridisation was performed in 50% formamide at 42°C as detailed by Higgins and Spencer (1991). The filters were washed twice with 2X SSC at room temperature for 15 min, followed by two washes in buffer containing 0.1% sodium pyrophosphate (NaPPi), 2X SSC, 0.1% SDS at 65°C for 15 min and a further 2 washes in a higher stringency buffer which contained 0.1% NaPPi, 0.1X SSC, 0.1% SDS at 65°C for 15 min The membranes were exposed to Fuji RX film at -70°C for 1-3 days with an intensifying screen, followed by fluorography.

2.2.5 Western blot analysis of transgenic plants

2.2.5.1 Detection of Na-PI

Freshly harvested tissues were ground with sand, using a mortar and pestle. Total soluble proteins were extracted using 5 ml of extraction buffer [100 mM Tris-HCl (pH 8.0), 2 mM $CaCl_2$, 10 mM ethylenediamine tetraacetic acid and 50 mM β-mercaptoethanol] per gm of tissue. Protein concentration was estimated by the method of Bradford (1976). Proteins (100 µg) were separated by SDS-polyacrylamide gel electrophoresis (15-25% gradient) (SDS-PAGE) according to Laemmli (1970) at 120V for 3 h. Relative molecular weights were estimated by reference to Bio-Rad prestained SDS-PAGE broad range markers. Purified Na-PI peptides from *N. alata* stigmas (Atkinson *et al.,* 1993a) were used as a positive control and for densitometric quantification (ImageQuant) of the amount of Na-PI peptides in the extracts from transgenic plants. A semi-dry transfer system (JKA-Biotech), modified from Laurière (1993), was used to transfer proteins from the gel (18 min, 12V) to nitrocellulose membrane (0.22 µm, Schleicher and Schuell). The transfer buffer contained 48 mM Tris-HCl, pH 9.4; 39 mM glycine and 20% (v/v) methanol. Proteins were fixed onto the

membrane by immersion in 100% isopropanol for one min, (M. Muskins, pers. comm.), followed by 12 min in 2% (v/v) glutaraldehyde (Sigma) (Karey and Sirbasku, 1989; M. Muskins, pers. comm.) in Tris-buffered saline (TBS). After washing for five min in TBS, the membrane was blocked for one h in TBS + 3% w/v bovine serine albumin (BSA) (Fraction V, Sigma). The membrane was washed for five min in TBS and incubated overnight at room temperature with a polyclonal rabbit antibody, specific to Na-PI (2 µg/ml protein A purified antibody diluted in TBS containing 1% w/v BSA) (Atkinson *et al.,* 1993a). The membrane was washed three times, each for five min, in TBS + 0.05% Tween-20 (TBST) + 0.1% w/v BSA and incubated with goat anti-rabbit IgG (Fc)-alkaline phosphatase conjugate (Promega) at room temperature for one h. After washing twice in TBST + 0.1% BSA and a final wash in TBS, alkaline phosphatase staining was performed according to manufacturer's instructions using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium reagent (Sigma).

2.2.5.2 Detection of intercellular Na-PI

To determine if Na-PI was secreted, the intercellular fluid was assessed for the presence of peptides which reacted with the Na-PI specific antibody. Three gm of leaf tissue (with midribs and veins removed) were placed in petri dishes containing 10 ml infiltration buffer [100 mM Tris-HCl (pH 8.0), 2 mM $CaCl_2$, 10 mM ethylenediamine tetraacetic acid, 50 mM β-mercaptoethanol and 0.5M sucrose], and infiltrated under vacuum for two min. Leaves were blotted dry on filter paper and placed into a 2.5 ml syringe barrel and the syringe and its contents were put inside a 15 ml Falcon tube. Intercellular fluid (IF) was collected by centrifuging the Falcon tube for ten min at 5000 rpm. Total protein was extracted from the remaining leaf by grinding in a mortar, and protein concentration was estimated as described in section 2.2.5.1. 100 µg of protein was concentrated with four volumes of cold acetone, precipitated for 30 min at -20°C, and recovered by centrifugation. The air-dried pellet was redissolved in protein loading buffer (Laemmli, 1970). SDS-PAGE and western blotting were as described above (section 2.2.5.1).

2.2.5.3 Detection of β-hordothionin

Protein extracts were prepared from 500 mg of tissue, as described in section 2.2.5.1 with the following additional steps. Ethanol was added to the protein extracts to a final concentration of 70%. The solution was incubated at room temperature for 30 min and clarified by centrifugation at 6 500 rpm for 10 min. The supernatant was transferred to a fresh tube and allowed to dry for 8 h in a Speed Vac concentrator (Savant). The pellet was dissolved in 100 µl of 0.1M SDS containing 1mM Tris, pH 7.0. Protein extracts were precipitated with bicinchoninic acid (Smith *et al.,* 1985) according to the supplier's instructions (Pierce, Rockford, USA) and aliquoted into microtitre plates (Greiner), which were read at 590 nm using a Multiscan Plus Microtitre plate reader (Labsystems). An estimate of protein concentration was calculated by Delta Soft II (a computer package which has an interface with Labsystems Multiscan Reader; version 4.3F). Fifty micrograms of protein were reduced in loading buffer containing 1% dithiothreitol (Progen Industries) and separated by SDS-PAGE as described by Schägger and Von Jagow (1987). All reagents and gels were prepared with Milli-Q Plus (Millipore) water. Rainbow™ coloured protein molecular weight markers (Amersham Life Science) were used to estimate the molecular mass of β-HTH. Gels were stained using SYPRO™ Red Protein Gel Stain (Molecular Probes). Immunoblotting was exactly as described for Na-PI (section 2.2.5.1) except for the omission of isopropanol fixation. The antibody with specificity to β-purothionin from wheat which cross-reacts with β-HTH (Carmona *et al.,* 1993a; Castagnaro *et al.,* 1994), was kindly made and supplied by Peter Hughes (CSIRO, Division of Plant Industry), and used at a 1:1000 dilution (~5 µg/ml). Peter Hughes also contributed equally to the detection of thionins in transgenic tobacco by western blotting.

2.2.6 Inheritance of genes in T_1 and T_2 progeny

The presence and expression of the *na-pi,* β-*hth* and *bar* genes was assessed in transgenic tobacco, pea and subclover, by self-pollinating the highest expressing primary transformants. Seed was collected from one β-HTH expressing line (#1); two tobacco Na-PI expressing lines (#13 and #24); one pea line (#10) and nine subclover lines. Thirty tobacco, 20 pea and 7 subclover seeds (T_1) from each of these lines were germinated in

soil. After four weeks, PPT (1 mg/ml for tobacco; 0.6 mg/ml for pea and 0.2 mg/ml for subclover) was applied to leaves with a paintbrush and plants were scored for tolerance or damage after seven days. Northern blot analysis of T_1 PPT-tolerant and PPT-sensitive plants was used to confirm phenotypes. The blots were probed with ^{32}P-labelled Na-PI or β-HTH DNA and T_1 plants which had approximately twice as much Na-PI or β-HTH mRNA as their siblings or parent plant were selected as potential homozygotes and self pollinated. Seeds were collected and germinated to produce the second generation (T_2). Homozygosity of the T_2 seedlings was confirmed by painting with PPT and northern blot analysis.

2.2.7 Crossing tobacco to generate double transformants

Two primary transgenic tobacco plants expressing the highest amount of foreign peptide (Na-PI line #24 and β-HTH line #1) were cross-bred to obtain individual plants which contained both genes. Immature tobacco flowers were emasculated (i.e., anthers were removed). Mature anthers from donor plants were excised and pollen was dusted on to moist stigmas of recipient plants. Reciprocal crosses were made to increase the chance of successful fertilisation. The entire flower was covered with a paper bag for two days and allowed to develop normally. Mature seed was selected and sown into a well draining soil, and after six weeks in the glasshouse, seedlings were assessed for tolerance to PPT as described in section 2.2.6.

2.3 Results

2.3.1 Plant transformation

Tobacco were transformed with *na-pi* or β-*hth* and 25and12 transformants, respectively, were selected for analysis of gene expression. For the legume transformation experiments, there were 12 putative pea transformants and 9 subclover plants chosen for molecular analysis.

Transformants from all three species were phenotypically normal when compared to untransformed plants (Fig. 2-3).

2.3.2 Analysis of Na-PI transcripts and peptides in transgenic plants

2.3.2.1 Tobacco

Na-PI transcripts in leaves of primary transformants

An mRNA species of 1.4 kb from leaves of T_0 transgenic tobacco hybridised to the Na-PI probe but was not present in untransformed plants (Fig. 2-4 A(i) and B(i)). The size of the observed transcript was the same as the size predicted from the open reading frame of the Na-PI cDNA. It was of sufficient size to code for the 43.3 kDa preproprotein of Na-PI detected in stigmas before cleavage of the secretion signal (Atkinson *et al.,* 1993a; Heath, 1994).

Variation in RNA loading and transfer was normalised by probing the same blots with 18S rRNA (Fig. 2-4A(ii) and B(ii)) and densitometric analysis was used to quantify the amount of Na-PI mRNA relative to 18S mRNA of the northern blots shown in A and B. The results of this analysis are presented in Fig. 2-4C(i) and (ii), respectively. The lines #2, #13 and #24 accumulated Na-PI mRNA to approximately similar levels that were over 4-fold higher than the average of the level in most other transformants.

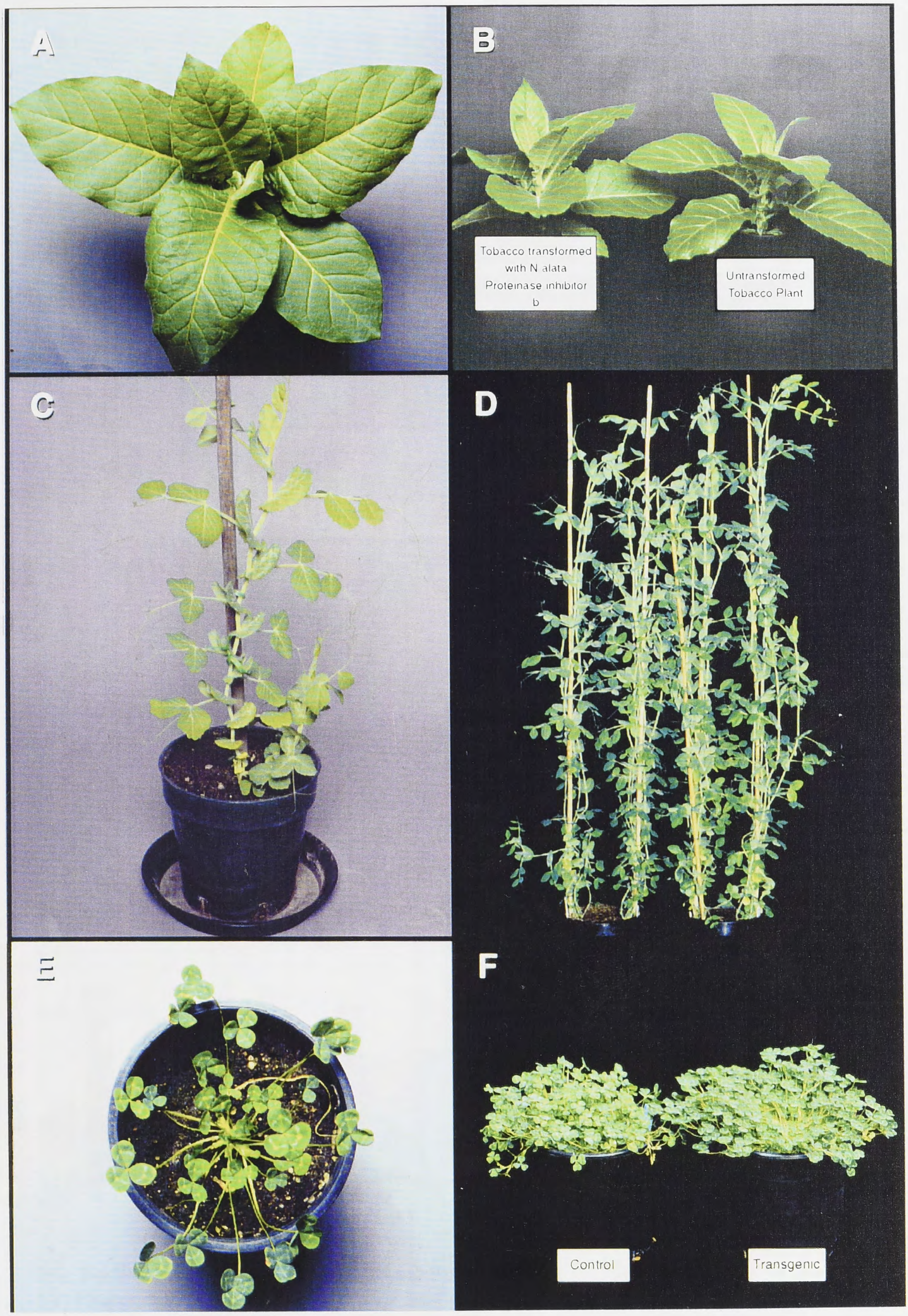


Fig. 2-3 Transgenic plants were phenotypically similar to control plants
(A) Transgenic tobacco were harvested for analysis when plants had reached a height of about 20 cm, with eight leaves. (B) comparison of transgenic tobacco (left) with untransformed tobacco (right). (C) transgenic peas had about 12 internodes and were about 40 cm in height when leaves were harvested for analysis. (D) mature transgenic peas relative (right) to untransformed controls (left). (E) leaves from transgenic subclover were harvested from plants of the age shown in this photograph. (F) mature transgenic subclover (right) compared to an untransformed plant (left). The phenotypes of transgenic plants were indistinguishable from untransformed control plants in all three cases.

Fig. 2-4 Northern analysis of transgenic tobacco containing the Na-PI chimeric gene (pAM8). (A) Fluorograph of a gel containing 5 µg (per track) total RNA from transgenic tobacco lines (#2-#13) and untransformed control (U). The number above the track represents an individual transgenic event. (i) Na-PI mRNA was detected by probing with a ^{32}P-labelled Na-PI cDNA and is arrowed. (ii) The same blot was probed with ^{32}P-labelled 18S ribosomal RNA to check for loading. (B) As described in (A) but analysis of transgenic lines #15-#25. (C) (i) and (ii) Quantification of the data presented in (A) and (B) respectively. The histogram shows the Na-PI counts normalised to the 18S rRNA counts. Na-PI specific mRNA was detected only in transgenic tobacco. The highest expressing lines were #2, #13 and #24.

Western blot analysis of T_0 transgenic tobacco

Total soluble leaf proteins from T_0 transgenic tobacco were separated by SDS-PAGE (Fig. 2-5A), and the level of proteinase inhibitor was determined by protein blot analysis (Fig. 2-5B), using an antibody raised against the 6 kDa PIs purified from *N. alata* stigmas (Atkinson *et al.,* 1993a). Na-PI-related polypeptides of M_r ~6000 were detected in transgenic tobacco leaves but were absent in leaves from untransformed controls (Fig. 2-5B(i) and (ii)). The amount of Na-PI polypeptides was estimated by comparison with 200 ng of Na-PI purified from stigmas (Fig. 2-5B (i); lane 1 and Fig. 2-5B (ii); lane 1, respectively). The level of Na-PI polypeptides in transgenic tobacco varied about 5-fold from 0.06 to 0.28% (lines #6 and #24, respectively) of total soluble protein (TSP). Except for line #4 and #21, high levels of Na-PI polypeptides correlated with high levels of Na-PI mRNA (compare Fig. 2-4 and Fig. 2-5). Total soluble protein from line #13 was loaded on both gels to enable comparison between all samples on the two western blots. Western blotting also showed the presence of a non-specific protein (M_r ~12 000) above the Na-PI-specific band (particularly evident in Fig. 2-5B(ii)). This band co-migrated exactly with the small subunit of Rubisco in the leaf proteins and its presence on western blots was probably due to a non-specific antibody binding, since it was also seen in untransformed controls.

Accumulation of Na-PI as a function of leaf-age

Leaves of increasing age were harvested from a T_0 tobacco plant line #24 which was ~35 cm tall, with fully expanded leaves, and was pre-flowering. Total soluble protein was separated by SDS-PAGE (Fig. 2-6A) and evaluated for the presence of Na-PI specific polypeptides (Fig. 2-6B). Na-PI accumulated to approximately 1.6% of TSP in the youngest leaf (Fig. 2-6B; leaf 1) and steadily decreased to 0.14% in mature leaves (Fig. 2-6B; leaf 11). The amount of Na-PI shown by western blotting, was also quantified by densitometry and calculated per gm of fresh weight (Fig. 2-6C(i)). The rationale was that 100 μg of TSP represented different amounts of tissue from each leaf age because total protein per leaf reached a peak at about leaf six and then declined (Higgins and Spencer, 1991). When expressed on a fresh weight basis the level of Na-PI also declined steadily with increasing leaf age (Fig. 2-6C(i)). This trend was quite different

from the pattern of total leaf protein accumulation which rose to a maximum per unit fresh weight in leaf six and then declined with increasing leaf maturity (Fig. 2-6C(ii)). It appeared, therefore, that Na-PI was less stable than the bulk of leaf proteins.

Distribution of Na-PI in different tissues of transgenic tobacco

A range of green and reproductive tissues were harvested from transgenic (line #24) and untransformed tobacco which were at equivalent developmental stages. Equal amounts of total soluble protein (100 μg) were fractionated by SDS-PAGE (Fig. 2-7A(i) and B(i)) and Na-PI levels estimated by immunoblotting (Fig. 2-7A(ii) and B(ii)) and on a fresh weight basis (Fig. 2-7A(iii) and B(iii)).

Na-PI-related polypeptides were detected in young and mature leaves (equivalent to leaf two and six, respectively, in Fig. 2-6B) of transgenic plants (Fig. 2-7A(ii)). There appeared to be an endogenous PI in mature leaves and stems of untransformed tobacco which cross-reacted with the Na-PI antibody. Despite this cross-reactivity, expression of peptides with Na-PI specificity was elevated in stems and petioles of transformed tobacco and was due to the presence of the transgene (Fig. 2-7A(ii)). Na-PI accumulation was also calculated on a fresh weight basis (Fig. 2-7A(iii) and B(iii)). In agreement with the results presented in Fig. 2-6, young leaves from transgenic plants contained more Na-PI per unit protein than mature leaves. Furthermore, in young or mature leaves, there was a 30-fold or 12-fold increase in Na-PI, over and above the presence of any endogenous, cross-reacting PI. On a fresh weight basis, the level of Na-PI in stems and petioles of transformed plants was only about a fifth of the amount which accumulated in mature leaves from these plants (Fig. 2-7A(iii)).

The sepals, pods and petals of both untransformed and transgenic tobacco contained peptides which reacted with the Na-PI antibody (Fig. 2-7B(ii)). Therefore, it was difficult to detect any additional Na-PI due to expression of the transgene, over and above the endogenous PI. The same was true, when the levels of Na-PI in reproductive organs were calculated on a fresh weight basis (Fig. 2-7B(iii)). In sepals, green pods and petals of transgenic plants, the Na-PI level was marginally higher than the amount of an

endogenous protein found in untransformed tissues, although any difference was likely to be insignificant. No such peptides were detected in seeds of transgenic or untransformed plants. The amount of Na-PI (ng/mg fresh weight) in sepals, green pods and petals of transgenic plants was much lower than the amount which accumulated in leaves (both histograms are drawn to the same scale, allowing direct comparison). Consequently, the leaves of transgenic plants are of the most interest to this thesis, as the presence of the Na-PI transgene contributed up to a 30-fold increase in the level of proteinase inhibitors.

Na-PI was confined to the intracellular space in transgenic tobacco

To determine if any Na-PI was localised extracellularly, intercellular fluid (IF) was extracted from leaves of T_0 transgenic tobacco line #24. Na-PI was detected by western blotting in the proteins of total leaf extracts, the intercellular fluid and extracts from the leaf which remained after IF extraction. On a fresh weight basis, the amount of Na-PI in the IF of transgenic leaves was negligible (Fig. 2-8), compared to the amount found in whole leaves or the remaining leaf, indicating that Na-PI was not secreted from the cell.

Inheritance of na-pi and bar genes in T_1 and T_2 progeny in transgenic tobacco

62% and 72% of T_1 tobacco seedlings from lines #13 and #24 were resistant to 1 mg/ml of PPT, respectively. An examination of PPT-tolerant and PPT-sensitive plants by northern analysis showed that only PPT-tolerant plants contained Na-PI mRNA. Furthermore, T_1 tobacco seedlings showed a two-fold range of Na-PI mRNA levels (Fig. 2-9T_1(i)). Tobacco lines #13.2 and #24.2 were estimated to contain twice the level as their siblings (after adjusting for differences in RNA loading). These lines were self-pollinated, and seeds were collected and germinated to generate T_2 plants. Na-PI mRNA accumulation was variable in the progeny from line #13.2 (Fig. 2-9 T_2(i)) and it was clear that this line was heterozygous. In contrast, the level of Na-PI mRNA in the T_2 tobacco seedlings derived from #24.2 were equal to each other (Fig. 2-9 T_2(i)) and about double the level of their T_0 parent (not shown). In addition, all T_2 progeny of line #24.2 were resistant to PPT. Furthermore, when T_2 progeny from line #24 were analysed for the presence of Na-PI polypeptides, the level of Na-PI was uniform (data not shown), so, taken together, line #24.2 appeared to be homozygous.

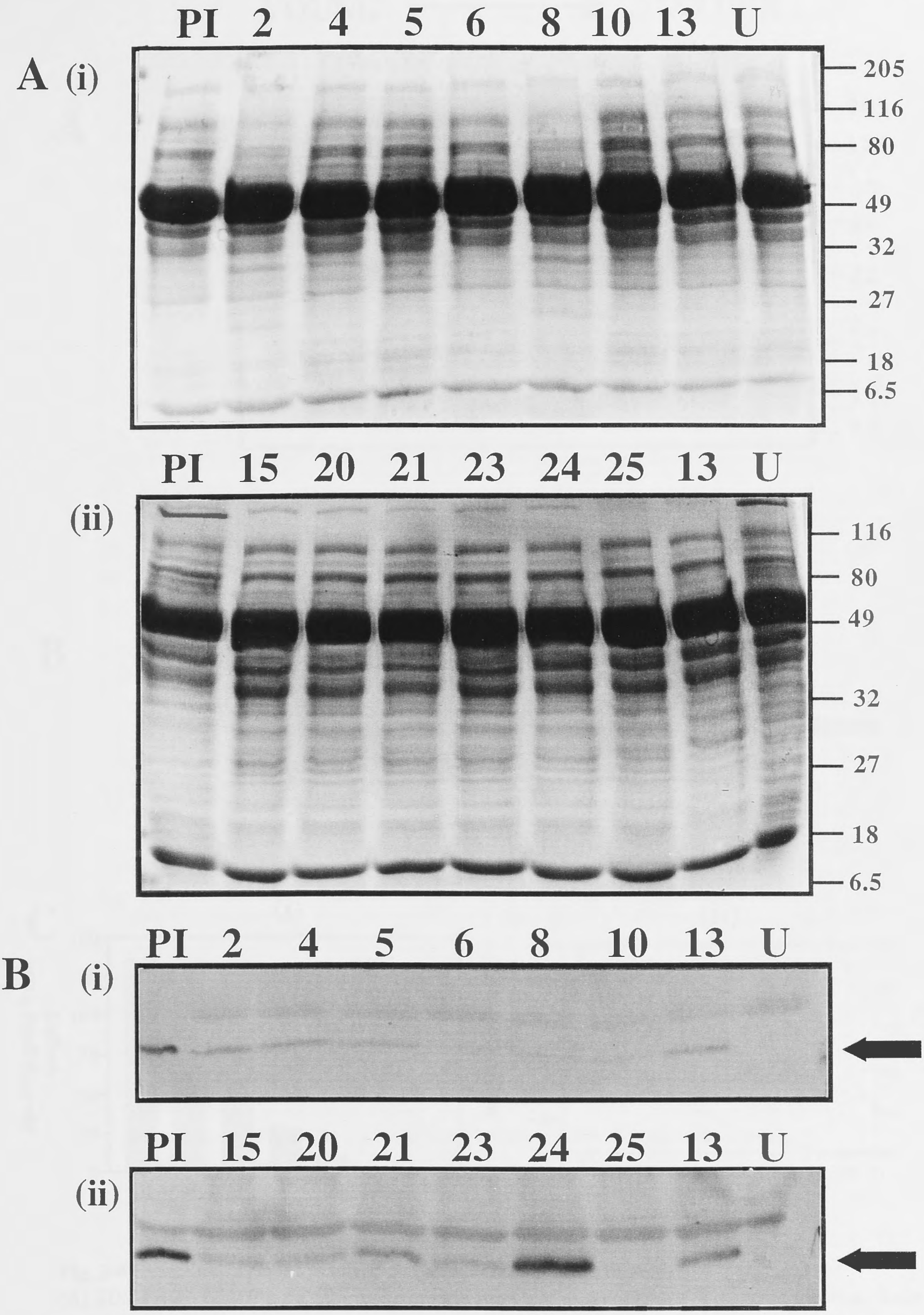


Fig. 2-5 Western blot analysis of transgenic tobacco containing chimeric Na-PI gene.
(A) (i) and (ii) Fractionation by SDS-PAGE of 100 μg total soluble protein from transformed tobacco lines #2-#25 as indicated by the number above the track. Untransformed plants are indicated by U and PI signifies 200 ng of purified Na-PI that had been spiked into 100 μg of total soluble protein from an untransformed control. The gels were stained with Coomassie Brilliant Blue. The size of the molecular weight markers (M_r 10^{-3}) are indicated on the right hand side of the gels. (B) Immunoblot probed with anti-Na-PI antiserum and detected by a secondary alkaline phosphatase-coupled antibody. Plant numbers are indicated above the blot. In leaves of transgenic tobacco the Na-PI precursor was cleaved into peptides of M_r ~6000. No Na-PI peptide was present in untransformed controls. The highest expressing lines were #13 and #24.

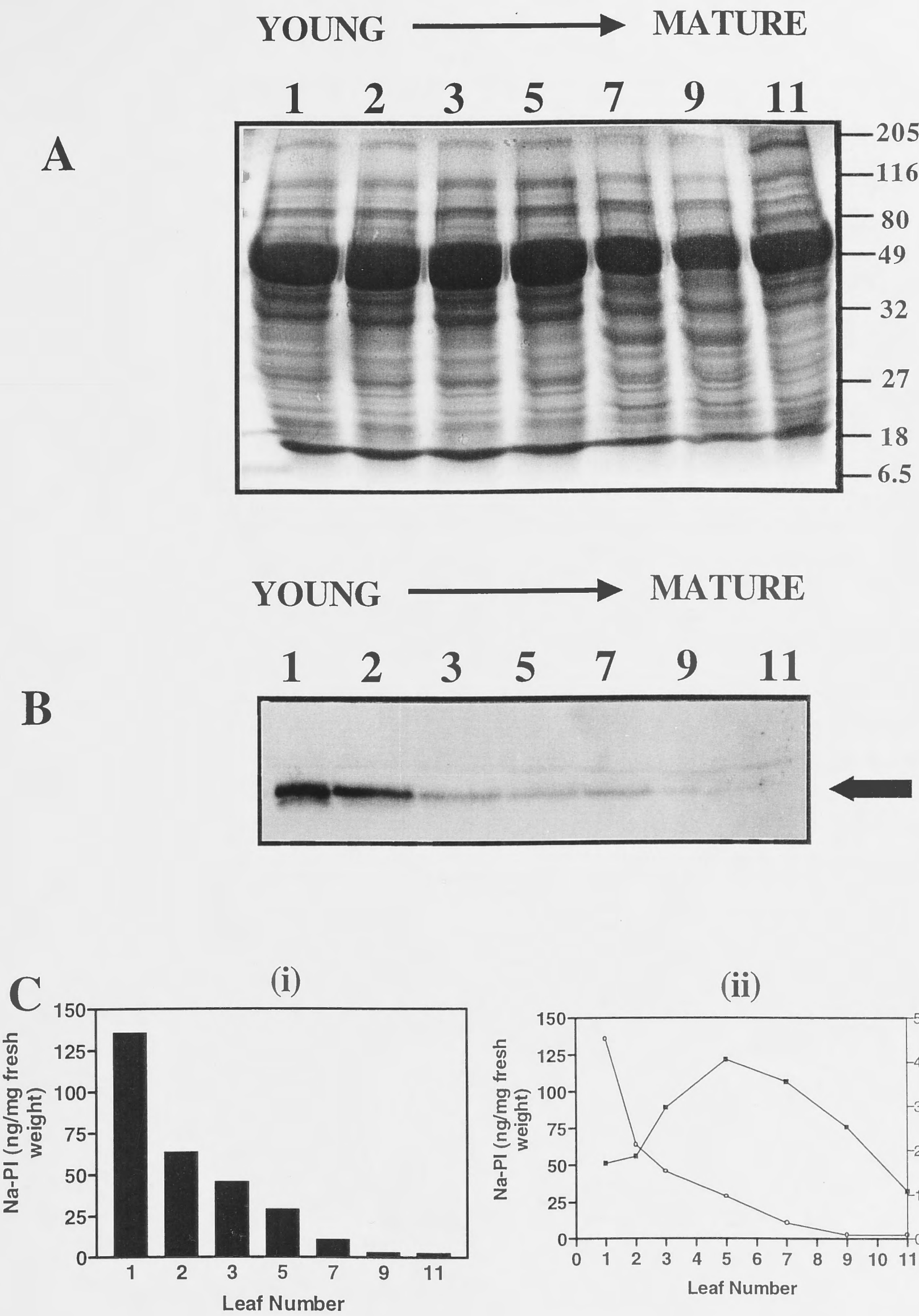
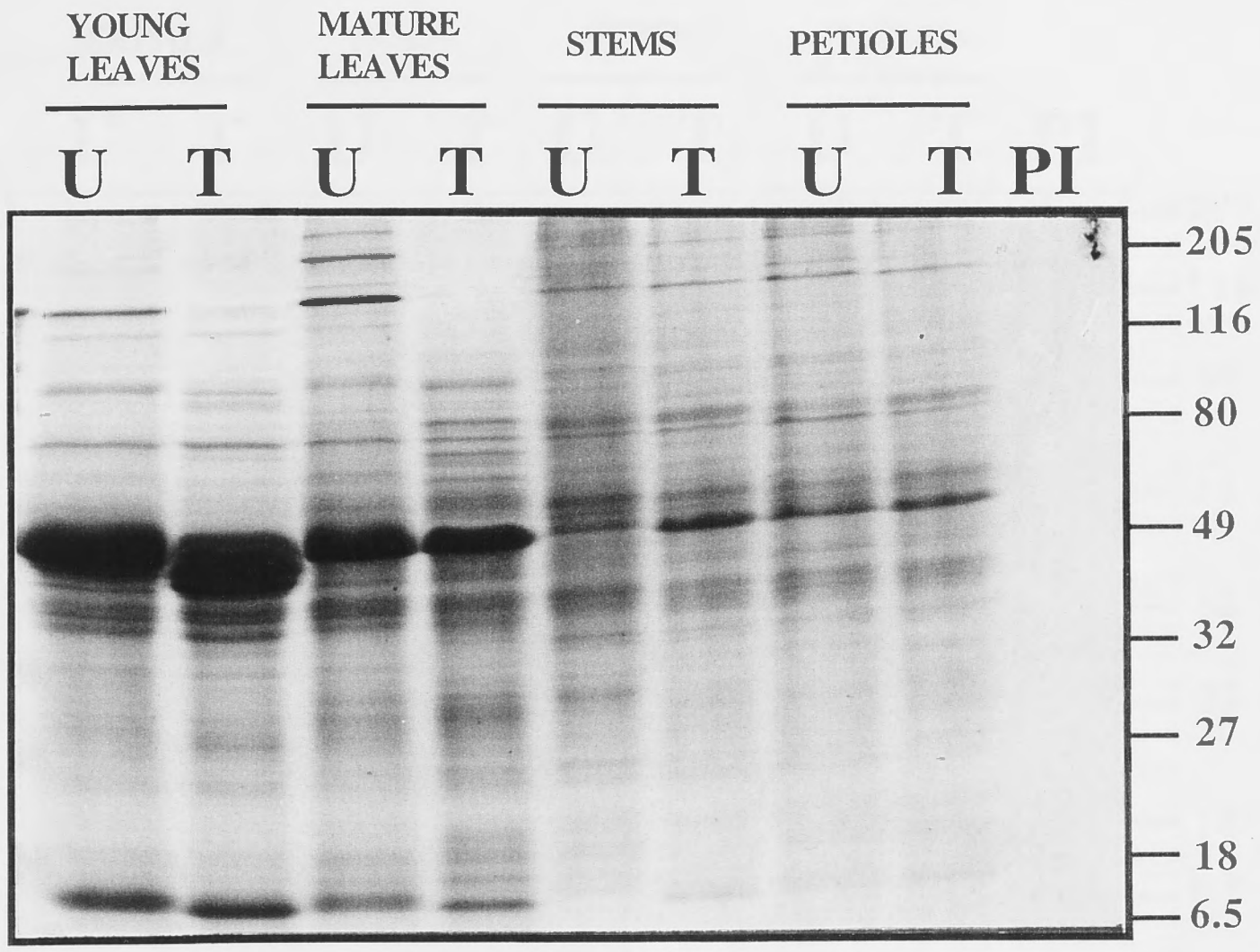


Fig. 2-6 Detection of Na-PI in a leaf-age series of transgenic tobacco

(A) SDS-PAGE of 100 µg total soluble protein from a leaf-age series of transgenic tobacco. Lane 1 contains protein from a newly opened leaf while 11 is from a mature senescing leaf. Relative molecular mass (M_r10^{-3}) is on the right hand side of the gel. (B) Immunoblot of (A); the nitrocellulose membrane was immunoreacted with a Na-PI-specific antibody. Na-PI is indicated by the arrow. (C) (i)Quantity of Na-PI on fresh weight (ii) Quantity of Na-PI on fresh weight basis (o—o) and total protein yield (■—■), both relative to leaf age. Na-PI accumulation decreased with increasing leaf age.

Fig. 2-7 Distribution of Na-PI in different tissues of T_0 transgenic tobacco, line #24
(A)(i) and (B)(i) total soluble proteins from different tissues of transgenic (T) and untransformed (U) tobacco fractionated by SDS-PAGE. The organ from which the protein originated is indicated above the track. All lanes contained 100 μg of protein. Lanes labelled PI each contained 250 ng of purified Na-PI and the size of the molecular weight markers are indicated on the right hand side of the gels (M_r 10^{-3}). A(ii) and B(ii) are western blots of the gels in (i), probed with anti-Na-PI antiserum and detected by a secondary alkaline phosphatase-coupled antibody. In A(iii) and B(iii), Na-PI is expressed in ng per mg fresh weight for each of the tissues in A(ii) and B(ii). Leaves of transgenic tobacco accumulated greater levels of Na-PI than other green tissues. Na-PI was not detectable in seeds.

A (i)


YOUNG LEAVES
MATURE LEAVES
STEMS
PETIOLES
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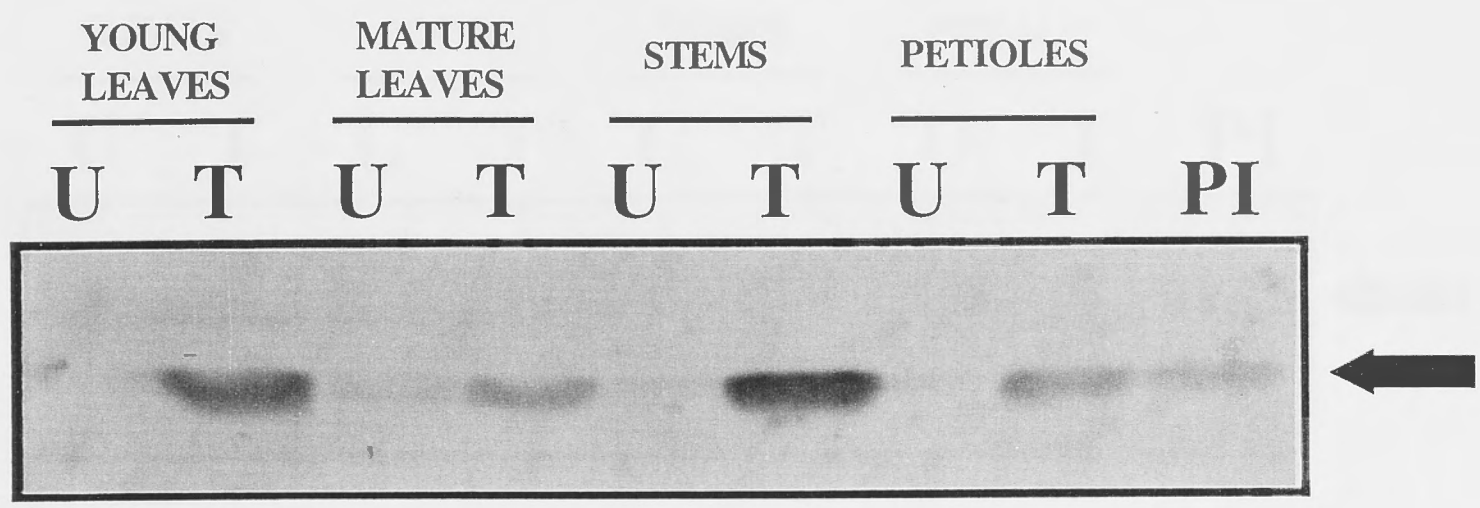
80
49
32
27
18
6.5

(ii)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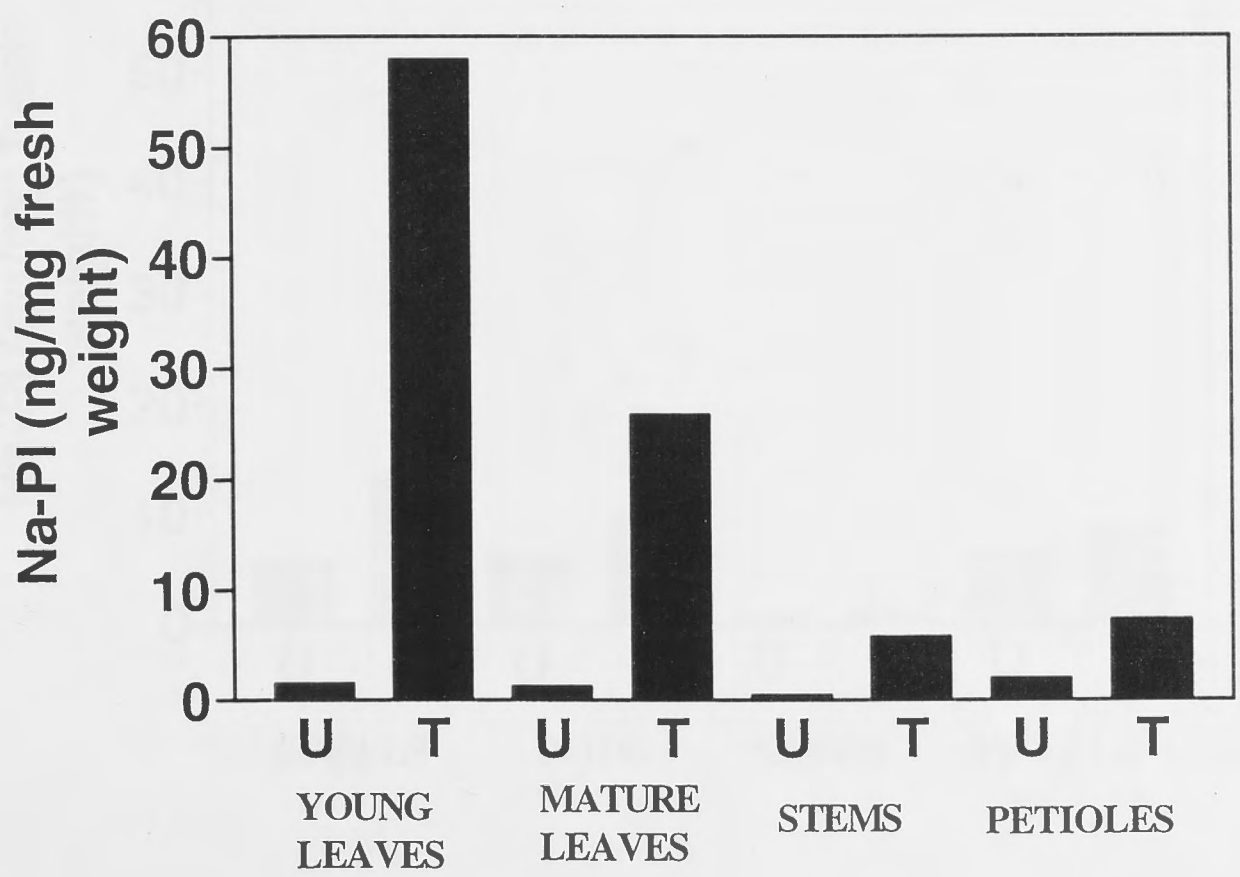
U T U T U T U T PI

(iii)


Na-PI (ng/mg fresh weight)
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50
40
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10
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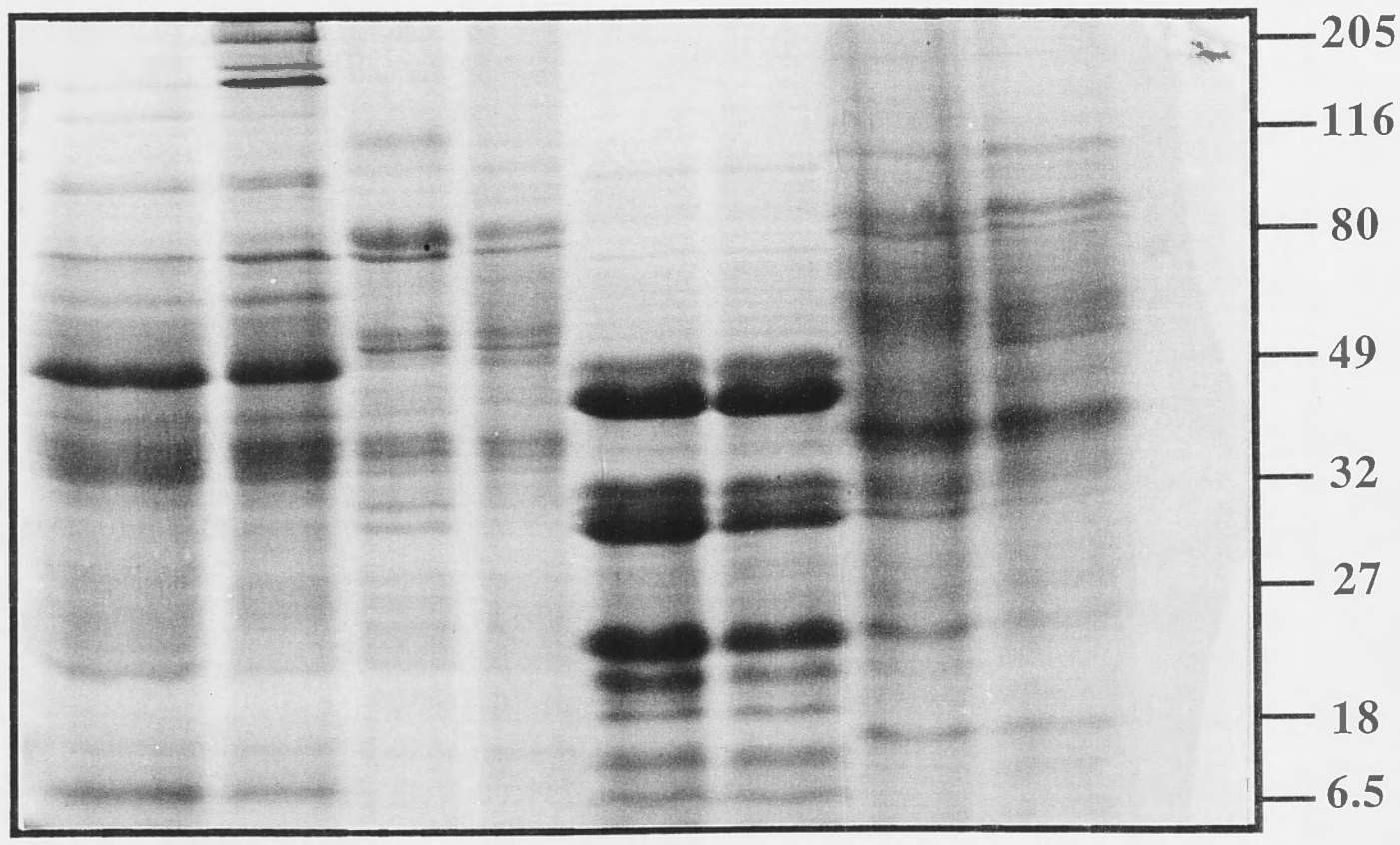
MATURE LEAVES
STEMS
PETIOLES

B

(i)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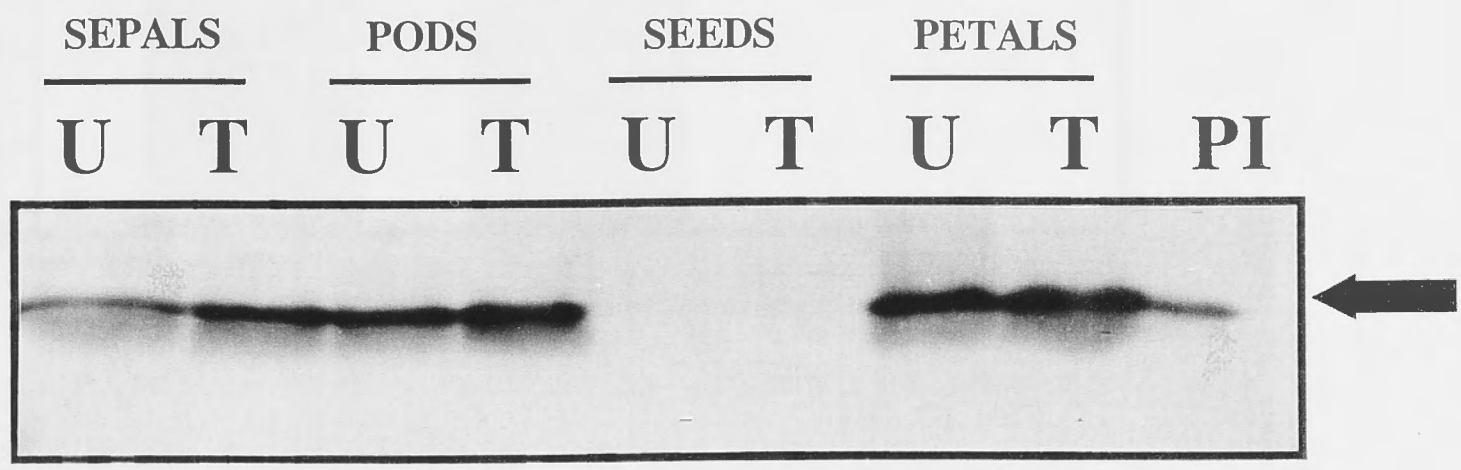
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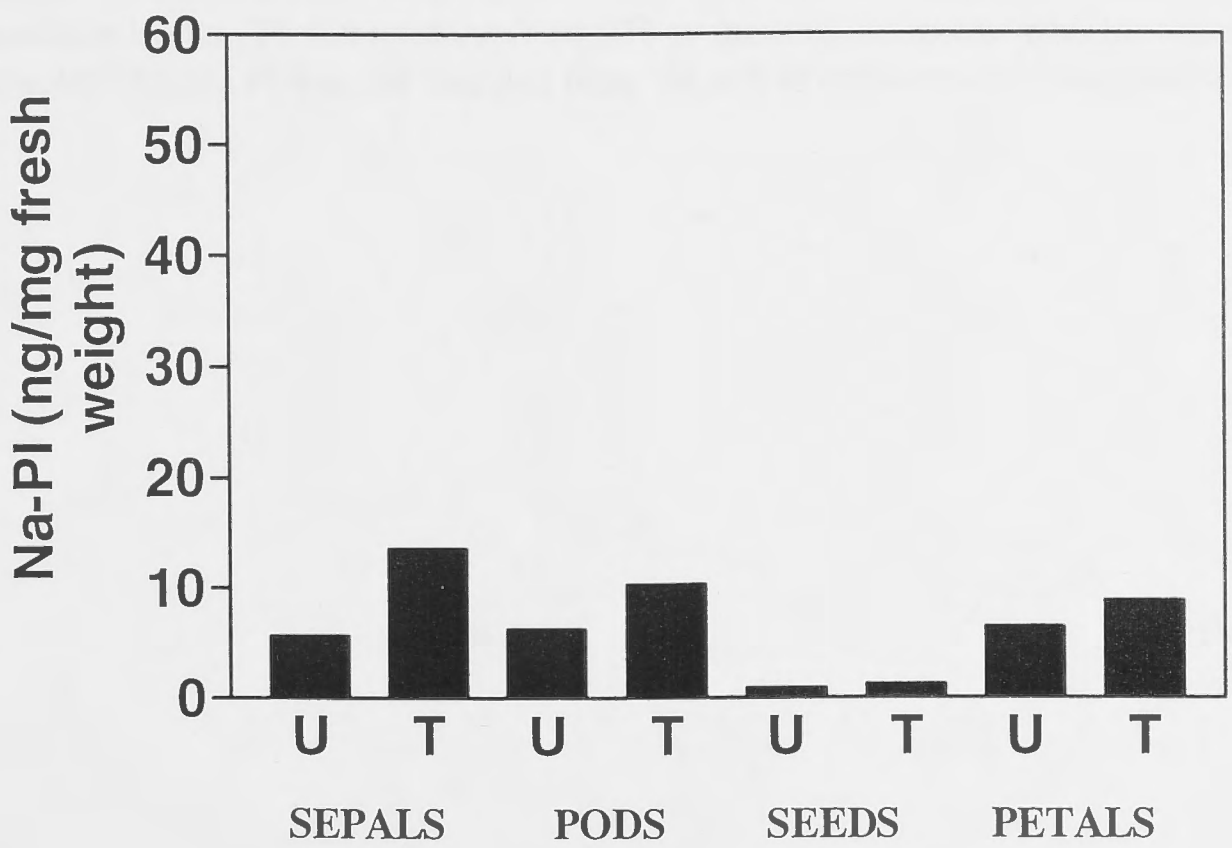
SEEDS
PETALS
U T U T U T U T PI

(iii)


Na-PI (ng/mg fresh weight)
60
50
40
30
20
10
0
U T U T U T U T
SEPALS
PODS
SEEDS
PETALS

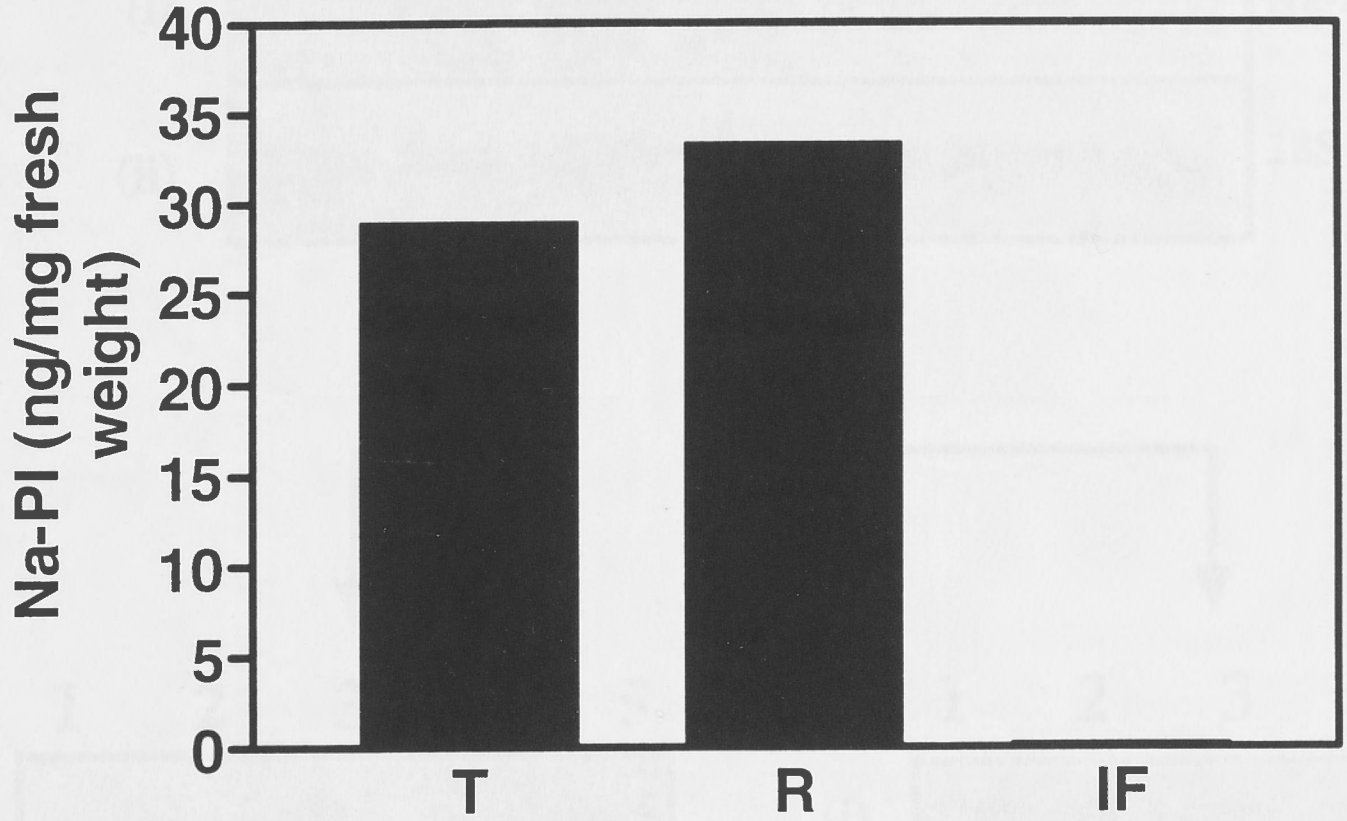


Fig. 2-8 Intracellular localisation of Na-PI in T_0 transgenic tobacco line #24
Quantification of Na-PI on a fresh weight basis. The extracts were labelled as follows: Total soluble protein from whole leaves (T), intercellular fluid (IF) or the remaining leaf after the intercellular fluid has been extracted (R). Na-PI was not secreted from the cell in the leaves of transgenic tobacco.

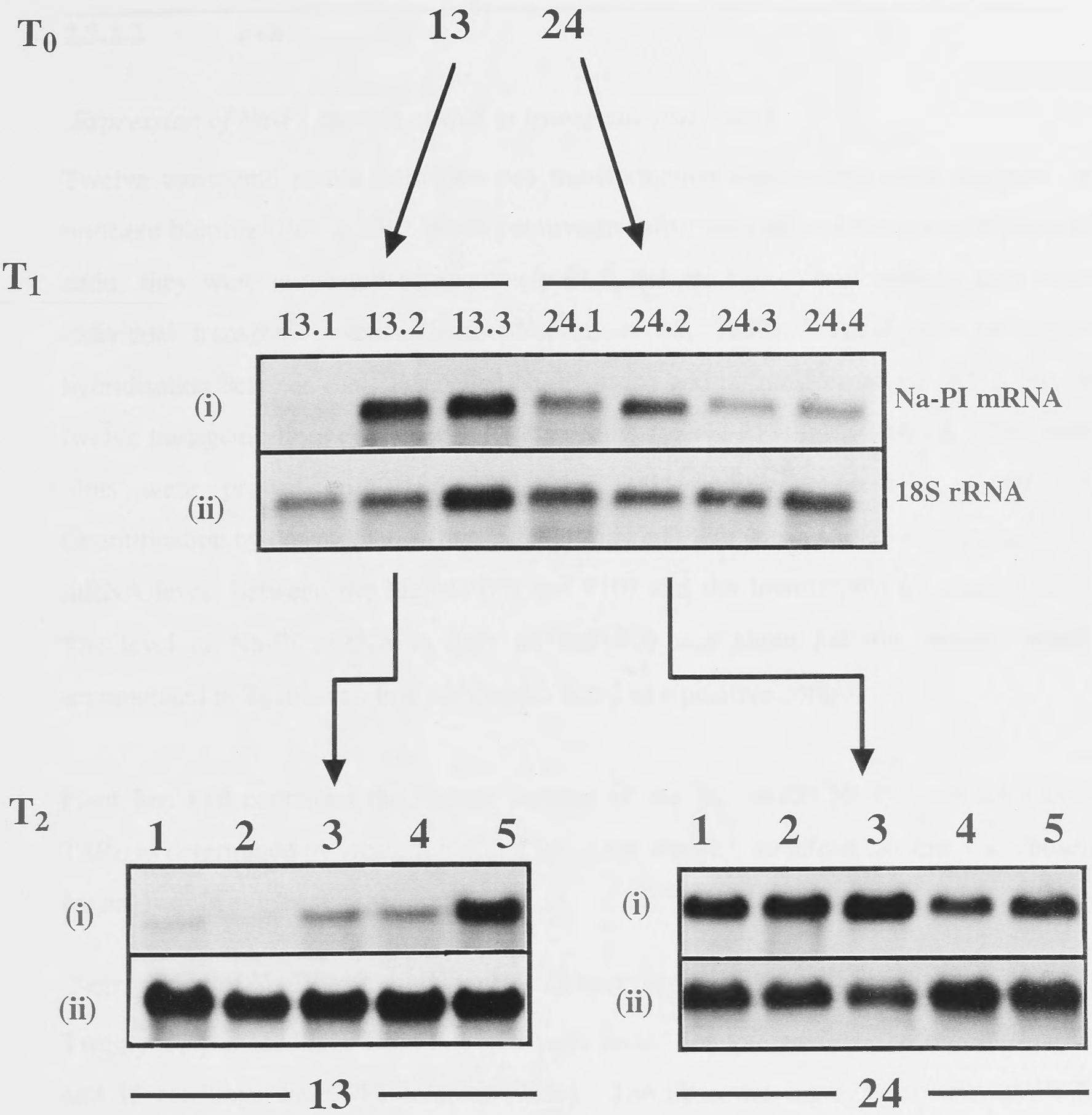


Fig. 2-9 Segregation of *na-pi* to T_1 and T_2 progeny in transgenic tobacco

The labels T_0, T_1 and T_2 on the left hand vertical axis are the primary transgenics, first and second generations respectively. (i) Na-PI mRNA was detected by probing 5 μg of total RNA with a ^{32}P-labelled Na-PI cDNA. (ii) The same blot was probed with ^{32}P-labelled 18S ribosomal RNA to check for loading. The numbers above the track in T_0 refer to the plant number and are the same as in Fig. 2-4 and Fig. 2-5. Downward arrows show the individual plant that was selected for analysis in the next generation. Na-PI was inherited to the T_2 generation. T_1 tobacco line #13.2 was heterozygous and line #24.2 was homozygous.

2.3.2.2 Pea

Expression of Na-PI specific mRNA in transgenic pea leaves

Twelve transgenic plants from two pea transformation experiments were analysed by northern blotting (Fig. 2-10). When putative transformants arose from a single piece of callus they were numbered consecutively (4.1, 4.2 etc.) as it was unlikely they were individual transgenic events (Schroeder, H. *et al.*, 1993). There was no cross-hybridisation between the ^{32}P-labelled Na-PI probe and untransformed pea RNA but all twelve transgenic lines contained mRNA specific for Na-PI (Fig. 2-10A(i)). The same blots were probed with 18S rRNA to enable normalisation (Fig. 2-10A(ii)). Quantification by densitometry (Fig. 2-10B) showed there was a twelve-fold variation of mRNA levels between the highest (#6 and #10) and the lowest (#8) expressing lines. The level of Na-PI mRNA in lines #6 and #10 was about half the amount which accumulated in T_0 tobacco line #24, which acted as a positive control (+).

Plant line #10 contained the highest amount of the M_r ~6000 Na-PI peptides (0.1% TSP), as determined by western blotting (data not shown), therefore this line was chosen for analysis in subsequent generations.

Segregation of Na-PI to T_1 and T_2 progeny in transgenic pea

Twenty T_1 pea seedlings from line #10 were tested for tolerance to 0.6 mg/ml of PPT and 15 seedlings were PPT-tolerant (75%). The observed segregation ratio of PPT-tolerant to PPT-sensitive T_1 seedlings was 3:1, providing evidence for transmission of the *bar* gene to subsequent generations as a dominant trait in these lines. As observed in tobacco, only plants resistant to PPT contained Na-PI mRNA, which indicated that the *bar* and *na-pi* genes were both active in these lines. The pea T_1 seedlings showed about a two-fold range of Na-PI mRNA levels (Fig. 2-11 T_1(i)). The pea line #10.4 which contained approximately twice the Na-PI mRNA level as its siblings (after adjusting for differences in RNA loading), was tested for homozygosity in the T_2 generation. In the T_2 seedlings derived from line #10.4, there was a uniformly high level of Na-PI mRNA (Fig. 2-11 T_2(i)). Likewise, all T_2 progeny of line #10.4 were resistant to PPT confirming that plants originating from this line were homozygous at the T-DNA locus

for the *bar* and *na-pi* genes. The level of Na-PI peptides in T_2 seedlings was approximately equal as well as being nearly double the amount found in the parent plant (data not shown).

2.3.2.3 Subterranean clover

Expression of Na-PI specific mRNA and peptides in transgenic subclover leaves

Northern analysis was used to detect Na-PI mRNA. The leaves from all lines of T_0 transgenic subclover analysed contained a mRNA species which hybridised to the Na-PI probe that was not present in untransformed plants (data not shown). The level of Na-PI polypeptides in transgenic subclover leaves varied about 8-fold from 0.025% to 0.2% of TSP (data not shown). Lines #4 and #6 were selected for analysis in T_1 and T_2 generations.

Segregation of Na-PI to T_1 and T_2 progeny in transgenic subclover

T_0 lines #4 and #6 were grown on to the T_1 generation. As for tobacco and pea, the observed ratios of PPT-tolerant to PPT-sensitive seedlings was close to the 3:1 segregation ratio expected for a dominant gene.

Only the PPT-tolerant plants were maintained in the glasshouse and analysed by northern blotting. On the basis of northern analysis (Fig. 2-12(i)), the two lines #4.1 and #6.1 were propagated to the second generation. All T_2 seedlings derived from these lines were tolerant to PPT and contained equal amounts of Na-PI mRNA (Fig. 2-12 T_2(i)). In addition, M_r ~6000 Na-PI peptides accumulated in T_2 transgenic subclover seedlings, and the amount of Na-PI peptides correlated well with mRNA quantification (data not shown). On the basis of this evidence, it appeared the T_1 lines # 4.1 and #6.1 were homozygous with respect to the transgene.

2.3.2.4 Interspecific comparison of levels of Na-PI

Total soluble protein extracts from the two highest expressing lines of transgenic tobacco, pea and subclover were separated by SDS-PAGE, and the level of Na-PI was determined by protein blot analysis. The western blot was repeated a total of three

times, using different extracts from young leaves of different T_2 plants and the western blot from one representative experiment is shown (Fig. 2-13A). Polypeptides of M_r ~6000 were detected in transgenic tobacco leaves but were absent in leaves from untransformed controls. Estimates of the level of Na-PI were made by comparison with 200 ng of Na-PI purified from stigmas and averaged for all the three experiments (Fig. 2-13B). The average level of Na-PI polypeptides in transgenic tobacco was estimated to be ~ 0.38% of TSP for tobacco lines, 0.08% for pea lines and 0.09% for subterranean clover, respectively.

The possibility that the lower levels of Na-PI in pea and subclover were due to other proteins in the leaf extracts masking Na-PI and thus reducing its detectability on a western blot was explored, but ruled out by a simple experiment. An equal amount of purified Na-PI from stigmas was spiked into a soluble protein extract from untransformed tobacco, pea and subclover, separated by SDS-PAGE and immunoblotted. The level of Na-PI in each extract was quantified by densitometry and found to be within 10% of each other (data not shown).

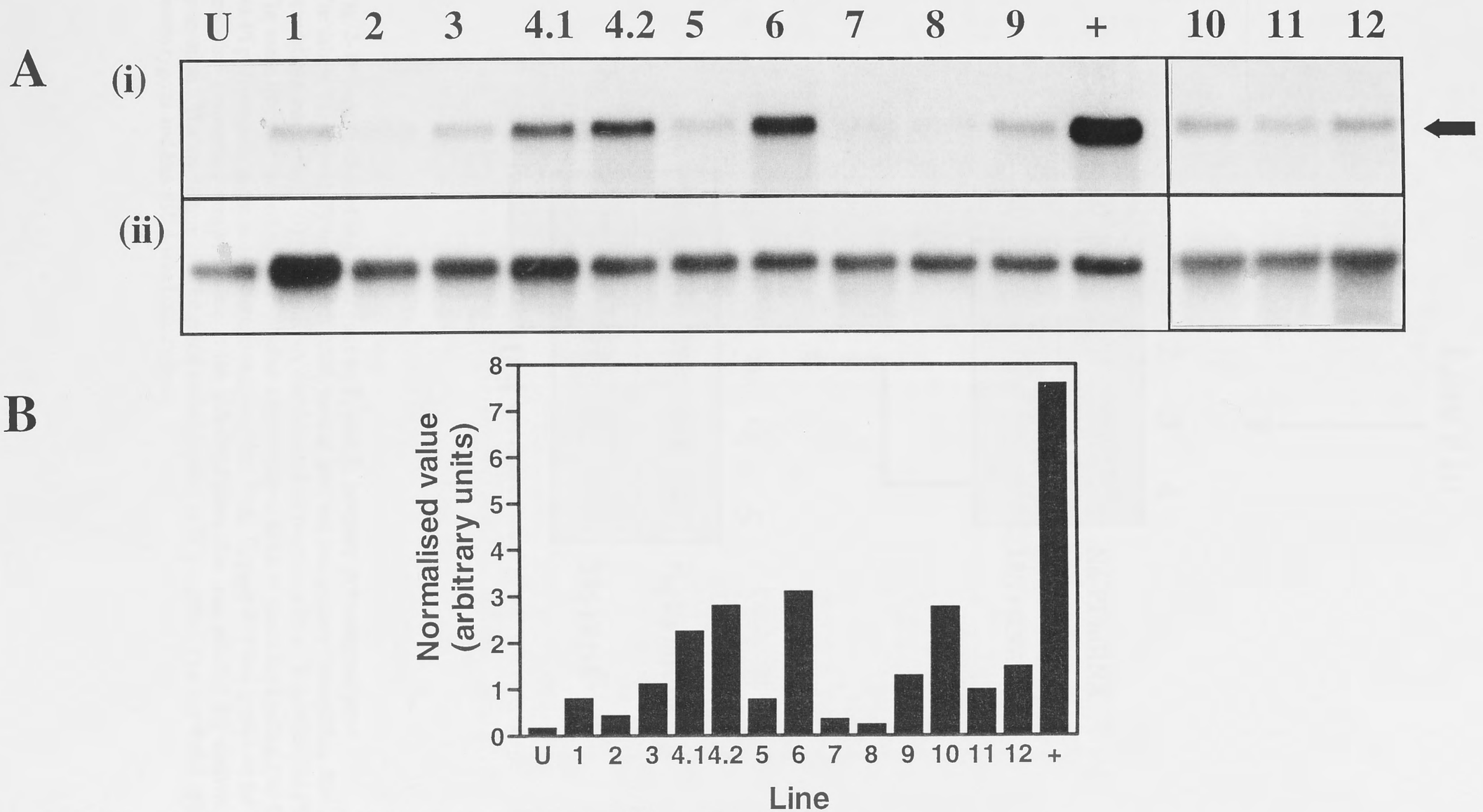


Fig. 2-10 Northern blot analysis of RNA from transgenic peas (T_0) containing Na-PI

(A) Fluorograph of two gels - each from separate transformation experiments. Each track contains 5 µg total RNA from transgenic pea lines (#1-#12) and untransformed control (U). '+' represents RNA from T_0 tobacco line #24, which acted as a positive control. The number above the track represents an individual transgenic event (unless otherwise stated). (i) Na-PI mRNA was detected by probing with a ^{32}P-labelled Na-PI cDNA and is arrowed. (ii) The same blot was probed with ^{32}P-labelled 18S ribosomal RNA to check for loading. (B) Quantification of the data presented in (A). The bars are Na-PI counts from the fluorograph in A(i) normalised to the 18S rRNA counts presented in A(ii). Na-PI mRNA was expressed in the leaves of transgenic pea. The highest expressors are lines #4.2, #6 and #10.

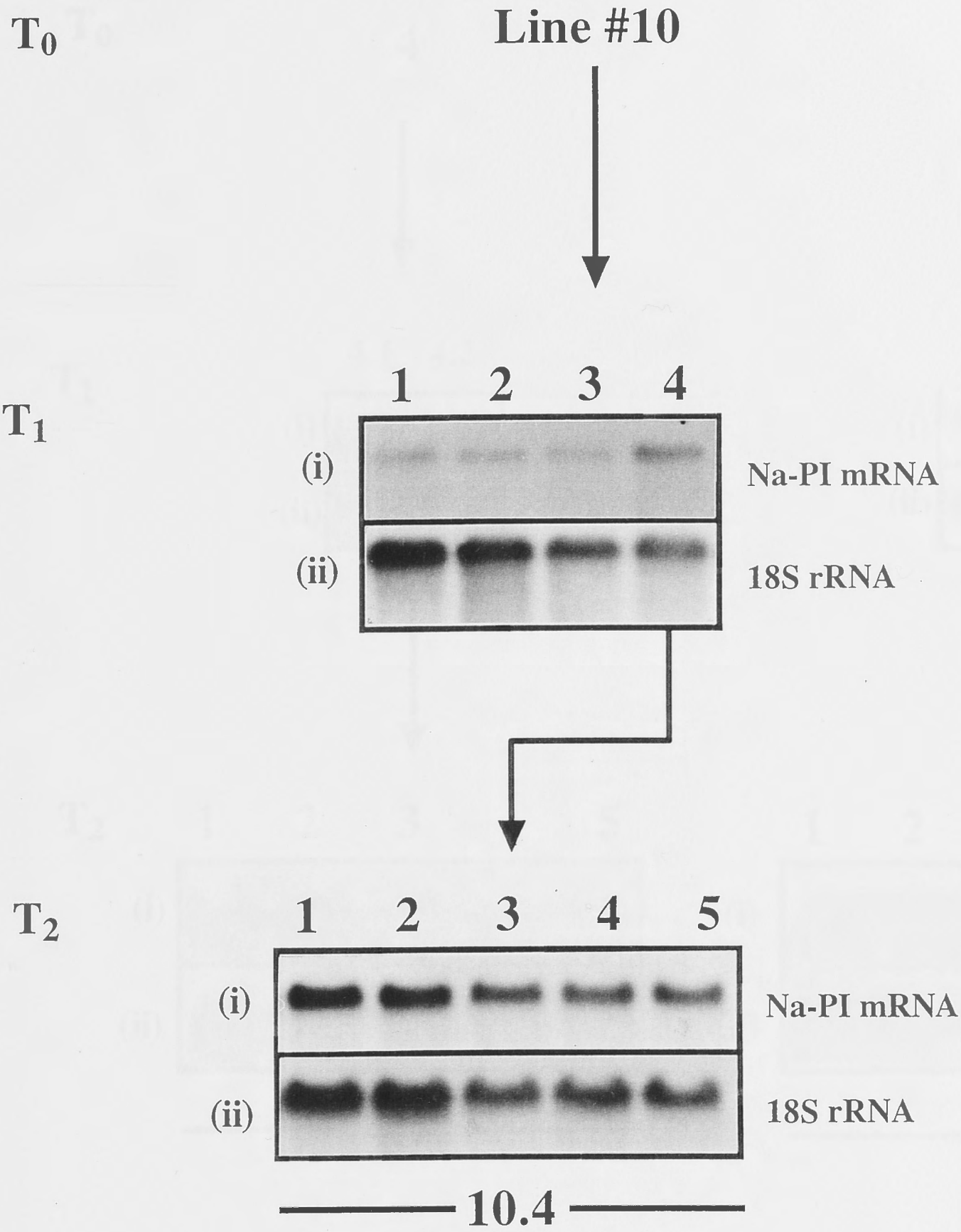
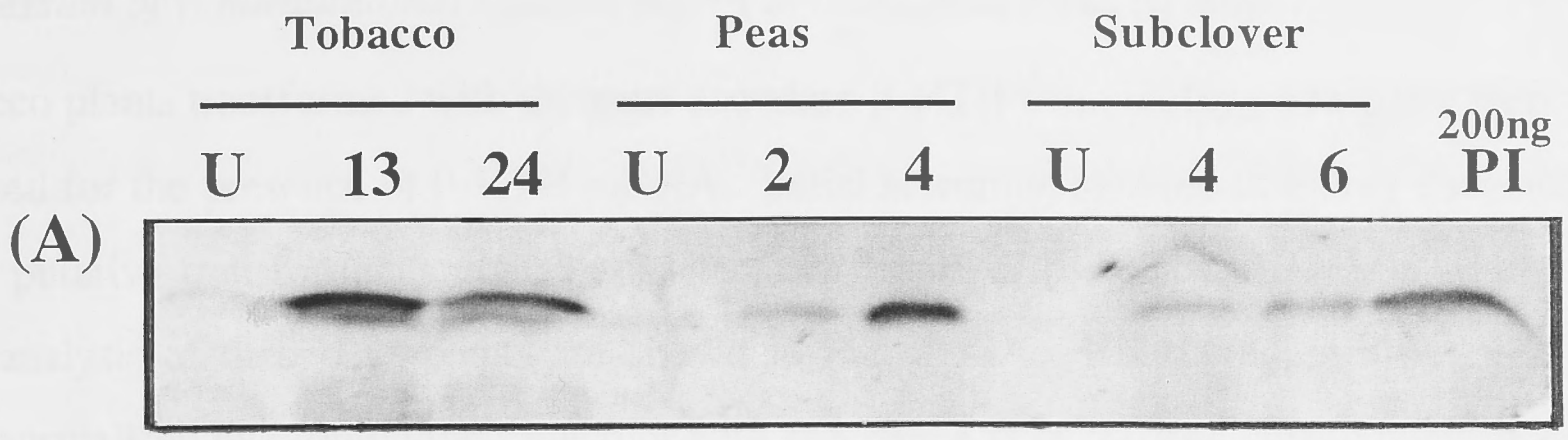


Fig. 2-11 Segregation of the *na-pi* gene to T_1 and T_2 progeny in transgenic peas
The labels T_0, T_1 and T_2 on the left hand vertical axis are the primary transgenics, first and second generations respectively. (i) Na-PI mRNA was detected by probing with a ^{32}P-labelled Na-PI cDNA. (ii) The same blot was probed with ^{32}P-labelled 18S ribosomal RNA to check for loading. (iii) Detection of Na-PI polypeptides in the same plants as in (i) and (ii). Only T_0 plant #10 was grown on for segregation analysis. Downward arrows initiate at the individual plant that was selected for analysis in the next generation. The *na-pi* gene was inherited and expressed in T_2 progeny. Pea line #10.2 appeared to be heterozygous and line #10.4 was homozygous.

Fig. 2-12 Segregation of the *na-pi* gene to T_1 and T_2 progeny in transgenic subclover
Primary transgenics, first and second generations are represented by the labels T_0, T_1 and T_2 respectively. (i) Na-PI mRNA was detected by probing 5 μg of total RNA with a ^{32}P-labelled Na-PI cDNA. (ii) The same blot was probed with ^{32}P-labelled 18S ribosomal RNA to check for loading. Only progeny from T_0 plants #4 and #6 were analysed. Downward arrows show the individual plant that was selected for analysis in the next generation. Two lines, #4.1 and #6.1 were homozygous at the Na-PI locus.



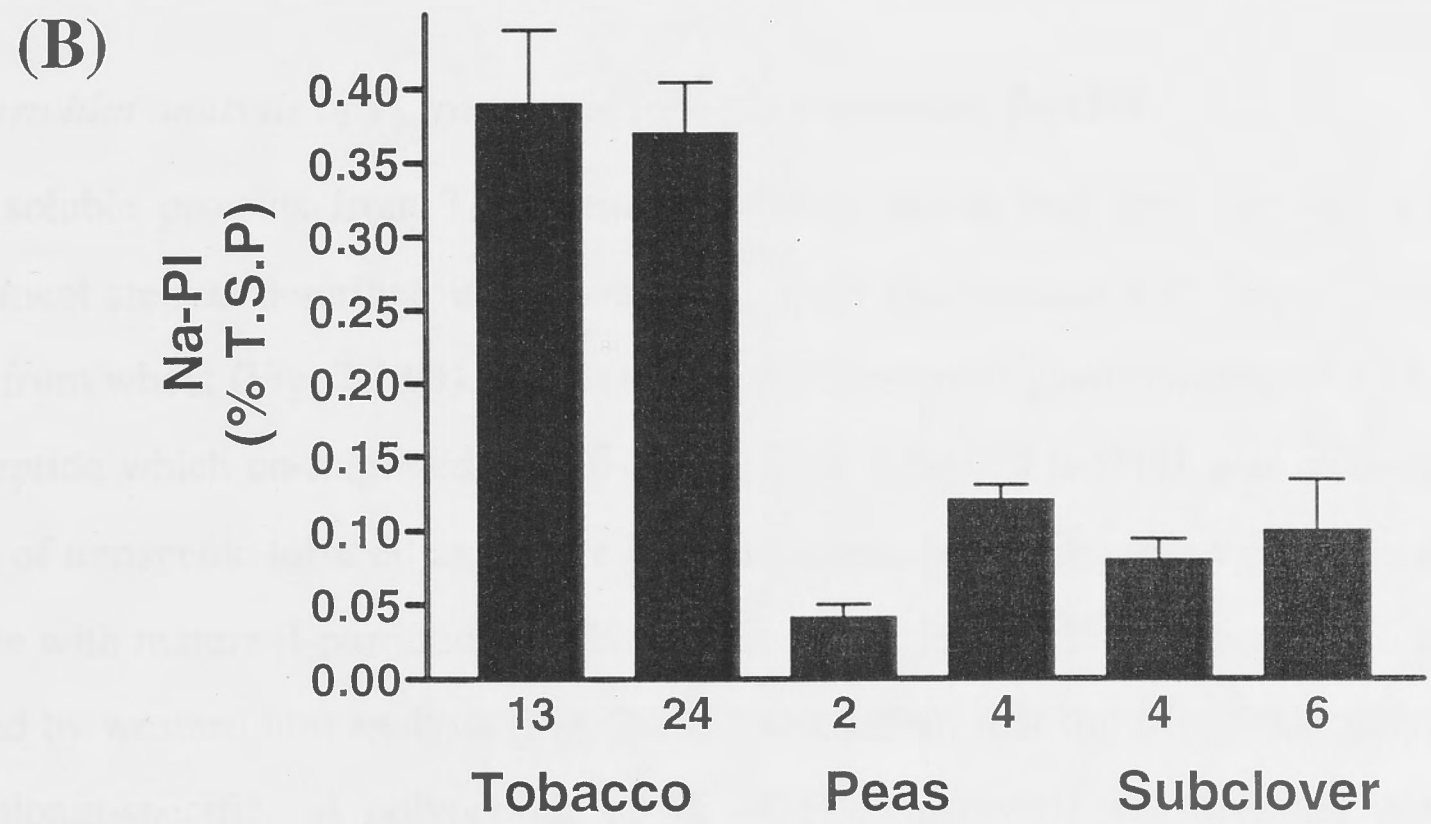


Fig. 2-13 Interspecific comparison of Na-PI expression levels in T_2 transgenic plants
(A) Protein (100 μg) from leaves of transgenic plants was fractionated by SDS-PAGE, and blotted onto nitrocellulose, probed with anti-Na-PI antiserum and detected by a secondary alkaline phosphatase-coupled antibody. The numbers above the track refer to the T_0 line from which T_2 plants were derived and U contains protein from an untransformed control of each plant species. One track contained 200 ng of purified Na-PI spiked into 100 μg of protein from an untransformed control. (B) Histogram of level of Na-PI peptides as a percentage of total soluble protein (TSP), estimated from the average of three experiments. The level of M_r ~6000 Na-PI peptides was higher in the leaves of transgenic tobacco, than in pea or subclover.

2.3.3 Analysis of thionin transcripts and peptides in transgenic tobacco

Expression of β-hordothionin specific mRNA in transgenic tobacco leaves

Tobacco plants transformed with the gene encoding β-HTH from barley endosperm were assessed for the presence of β-HTH mRNA. Initial screening showed that only five out of 12 putative transformants contained detectable levels of β-HTH mRNA and results from analysis of these five plants are shown in Fig. 2-14A. RNA loading differences were normalised by probing the same blot with 18S rRNA (Fig. 2-14A(ii)) and quantified by densitometry (data not shown). Plant #1 had the highest level of mRNA, so progeny of this line were further analysed. The ^{32}P-labelled β-HTH DNA probe did not hybridise to RNA from an untransformed control.

Western blot analysis of T_0 transgenic tobacco containing β-HTH

Total soluble proteins from T_0 transgenic tobacco which had been through a thionin enrichment step as described in section 2.2.5, were fractionated with β-purothionin (β-PTH) from wheat (Fig. 2-14B). An extract from transgenic plants contained a M_r ~7000 polypeptide which co-migrated with β-PTH. This indicated β-HTH was cleaved in the leaves of transgenic tobacco as mature β-hordothionin had been shown previously to co-migrate with mature β-purothionin (Castagnaro *et al.,* 1994). Four transgenic lines were assayed by western blot analysis (Fig. 2-14C) to confirm that the Mr ~7000 polypeptide was thionin-specific. A polypeptide of M_r ~8 500 (arrowed) was detected in all four transgenic tobacco lines but not in the untransformed control. The larger proteins (M_r ~ 40 000 and ~32 000) which cross-reacted with the antibody were likely to be non-specific as these were also detected in untransformed controls. Purified β-HTH from barley endosperm was not available, so the level of β-HTH in transgenic tobacco leaves was not quantified.

Segregation of β-HTH in T_1 and T_2 progeny of transgenic tobacco

Seventy percent of T_1 tobacco seedlings containing β-HTH derived from line #1 were resistant to 1 mg/ml of PPT. The observed segregation ratio of PPT-tolerant to PPT-

sensitive seedlings was close to the expected 3:1 ratio, and given the small sample size (n = 30), it appeared that the *bar* gene was inherited by T_1 plants as a dominant Mendelian trait. Northern blot analysis revealed that only PPT-tolerant plants contained β-HTH mRNA, confirming that the T-DNA construct consisting of the *bar* and β-*hth* genes were both expressed from a single locus in the tobacco genome. Tobacco line #1.3 which contained more β-HTH mRNA than its siblings (after adjusting for differences in RNA loading), was taken through to the T_2 generation. β-HTH mRNA accumulation was variable in the five tested progeny from this line (Fig. 2-15T_2 (i)) although, as all 30 T_2 seedlings screened were resistant to PPT, each plant probably contained β-HTH mRNA (because both *bar* and β-*hth* genes segregated as a single unit). Therefore homozygosity was assumed for the transgenic locus but with variable levels of β-HTH expression. One explanation is that the original transformant may have had more than one copy of the transgene or alternatively, as for *na-pi*, the different age of the leaves sampled for RNA isolations may be the reason for this variation.

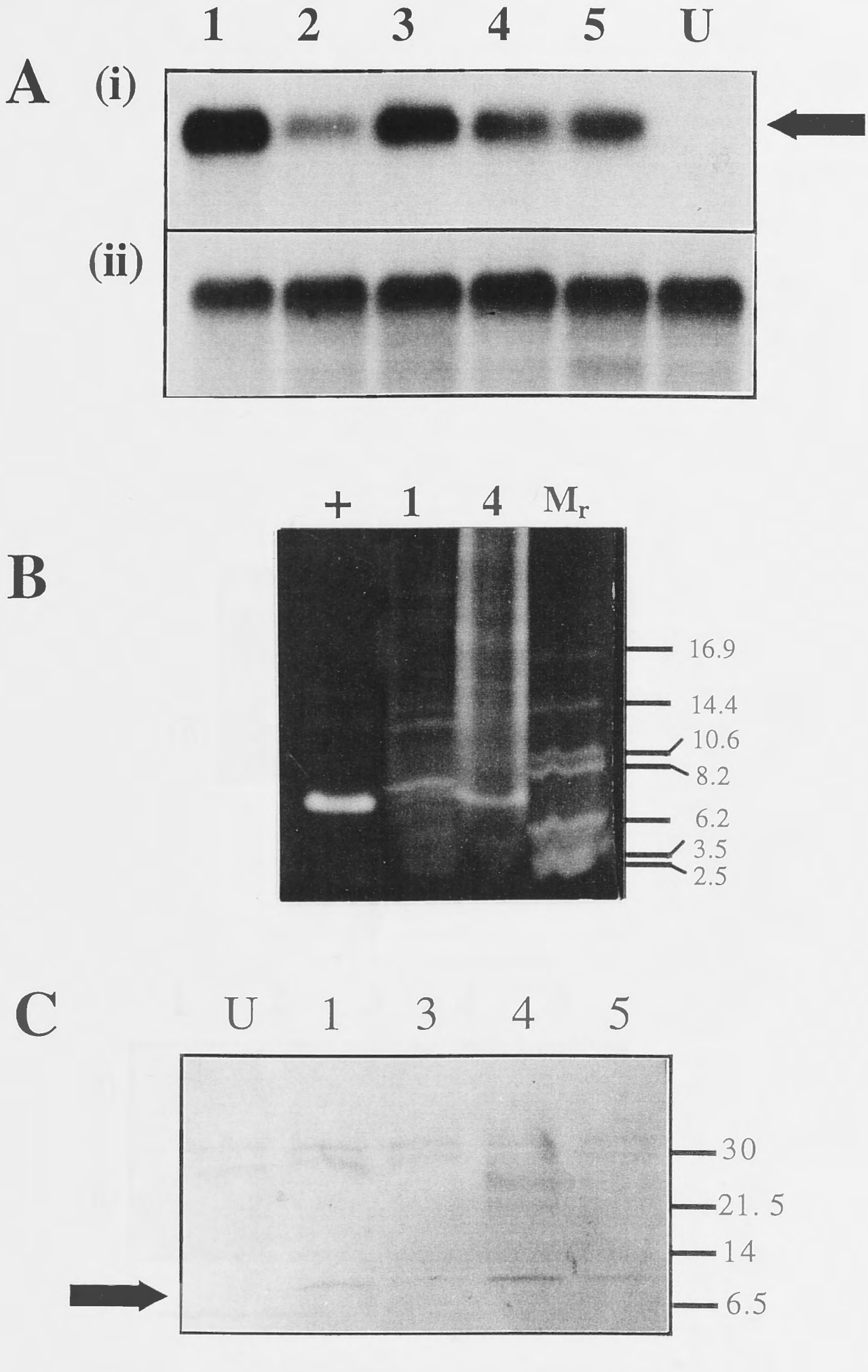


Fig. 2-14 Northern and western analysis of tobacco transformed with β-hordothionin
(A) Total RNA (5 μg per lane) was fractionated by electrophoresis, blotted onto nitrocellulose and mRNA was detected by fluorography after probing with (i) ^{32}P-labelled β-HTH cDNA or (ii) a ^{32}P-labelled 18S ribosomal RNA gene fragment. The numbers above the track contain RNA from individual transformed tobacco plants and U contains RNA from an untransformed control. (B) Fractionation by SDS-PAGE of 15 μg of leaf extracts from transgenic tobacco lines #1 and #4, which had been enriched for thionins. + signifies ~130 ng of purified β-purothionin from wheat which acted as a positive control. The gel was stained in SYPRO™ red protein gel stain. The size of the molecular weight markers (M_r 10^{-3}) are indicated on the right hand side of the gel and are also shown as there was some distortion. (C). Immunoblot of thionins extracted from transgenic plants and probed with anti-β-purothionin. Plant numbers are indicated above the blot. In leaves of transgenic tobacco the β-hordothionin precursor was cleaved into peptides of M_r ~8500. No β-hordothionin was present in untransformed controls.

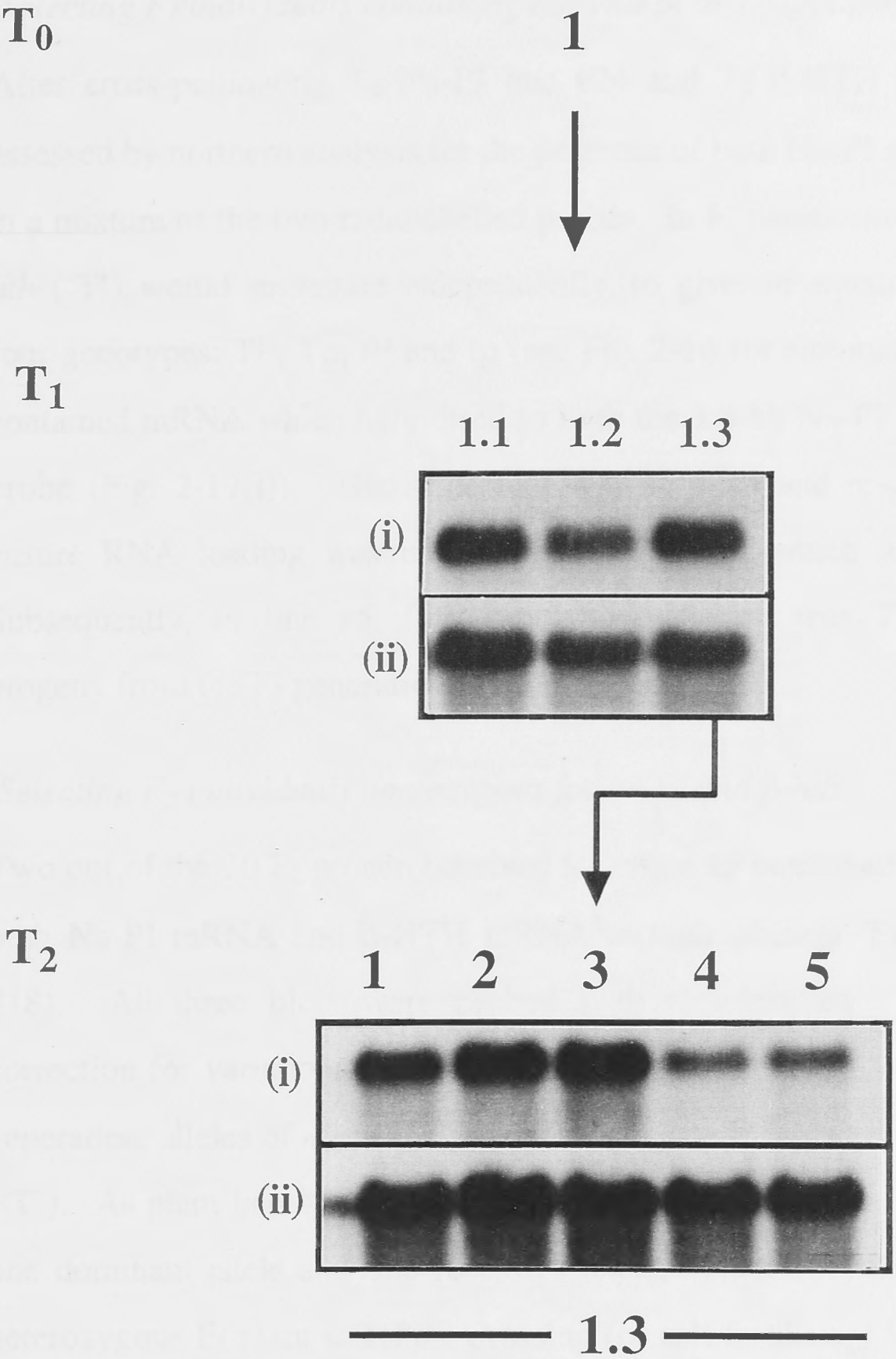


Fig. 2-15 Segregation of β-hordothionin gene to T_1 and T_2 progeny in transgenic tobacco by northern blotting

T_0, T_1 and T_2 on the left hand vertical axis refer to the primary transformants, first and second generations, respectively. Only progeny from T_0 line #1 were analysed by northern blotting. (i) fluorograph of an RNA blot probed with β-hordothionin cDNA and (ii) the same blot probed with 18S rRNA to check for RNA loading. The downward arrows initiate at the individual plant that was selected for analysis in the next generation. β-HTH mRNA was inherited to the T_2 generation. The line #1.3 was homozygous with respect to the transgene as all T_2 progeny were tolerant to PPT.

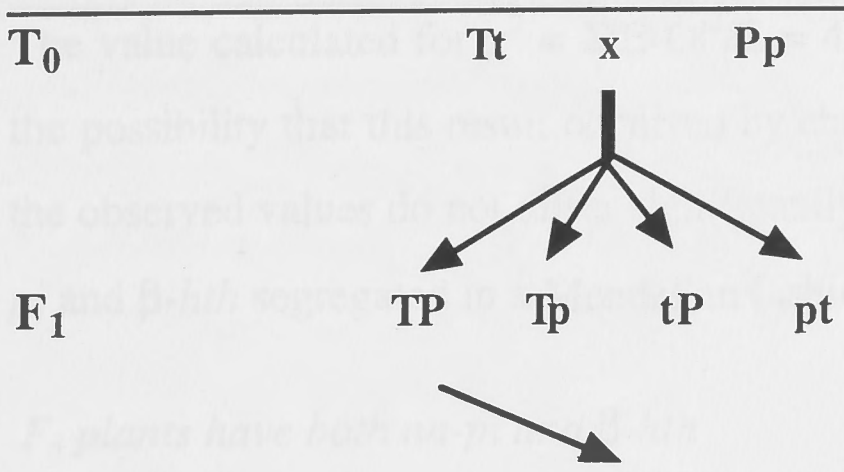
2.3.4 Analysis of double transformants

Selecting F_1 individuals containing both na-pi and β-hth genes

After cross-pollinating T_0 Na-PI line #24 and T_0 β-HTH line #1, F_1 seedlings were assessed by northern analysis for the presence of both Na-PI and β-HTH by hybridisation in a mixture of the two radiolabelled probes. In F_1 plants, *na-pi* (abbreviated 'P') and β-*hth* ('T') would segregate independently, to give, in equal proportions, the following four genotypes: TP, Tp, tP and tp (see Fig. 2-16 for summary). Two lines (#4 and #5) contained mRNA which hybridised to both the 1.4 kb Na-PI probe and the 1 kb β-HTH probe (Fig. 2-17(i)). The same blot was stripped and re-probed with 18S rRNA to ensure RNA loading was uniform, the results of which are shown in Fig. 2-17(ii). Subsequently, F_1 line #5, (the genotype of which was TP), was self-fertilised and progeny from the F_2 generation were analysed.

Selecting F_2 individuals homozygous for na-pi and β-hth

Two out of the 30 F_2 progeny derived from line #5 contained nearly twice the amount of both Na-PI mRNA and β-HTH mRNA as their siblings (Fig. 2-18; A(i) #8 and A(ii) #18). All three blots were probed with radiolabelled 18S rRNA to permit correction for variations in RNA loading and transfer (Fig. 2-18; lower blot). In the F_2 generation, alleles of *na-pi* ('P') should segregate independantly from the alleles of β-*hth* ('T'). As plant line #5, was shown to contain both defence genes, each gene will have one dominant allele and one recessive allele. Therefore, the correct genotype for the heterozygous F_1 plant is TtPp. Crossing (or self-fertilising) this heterozygous individual produces the F_2 generation with the expected 9:3:3:1 ratio of phenotypes (cited in Raven and Johnson, 1989). Nine out of the total of 16 plants represent the proportion of F_2 progeny that will show the two dominant characteristics, 1/16 will show the two recessive characteristics and 3/16 (x2) will show the two alternative combinations of dominants and recessives (Fig. 2-16).



	TP	Tp	tP	tp
TP	TTPP	TTPp	TtPP	TtPp
Tp	TTPp	TTpp	TtPp	Ttpp
tP	TtPP	TtPp	ttPP	ttPp
tp	TtPp	Ttpp	ttPp	ttpp

TP : T__ : __P : __ __

9 : 3 : 3 : 1

Fig. 2-16 Segregation of *na-pi* (P) and β-*hth* (T).
T_0 primary transgenics containing either Na-PI or β-HTH were cross-fertilised to produce four phenotypes of F_1 progeny. A plant containing both genes (TP) was selfed. As the two genes would segregate independently, the genotypes of the T_2 progeny will, on average, be in the ratio of 9:3:3:1.

If the hemizygotes are not considered, taken simply, four F_2 RNA genotypes: TP, T__, __P and __ __ were expected. Assuming that the expected ratio of the F_2 phenotypes is 9:3:3:1, a χ^2 test will test the hypothesis that the expected ratio is not significantly different to the observed ratio. A summary of the RNA genotypes of the 30 seedlings is given below (Table 2-1).

Table 2-1 Segregation ratio of F_2 progeny derived from Na-PI/β-HTH line #5

RNA GENOTYPE	TP	T__	___P	___ ___
Expected (E) proportion*	16.9	5.6	5.6	1.9
Observed (O) proportion	15.0	9.0	6.0	0
|E-O|	1.9	3.4	0.4	1.9
$\|E-O|^2$	3.6	11.56	0.16	3.61
$\|E-O|^2/E$	0.21	2.06	0.03	1.9

*proportion of 30, calculated from the expected ratio 9:3:3:1.

The value calculated for $\chi^2 = \Sigma|E\text{-}O|^2/E = 4.2$; ($P < 0.15$). With 3 degrees of freedom, the possibility that this result occurred by chance alone was about 15%. This means that the observed values do not differ significantly from the expected values and therefore *na-pi* and β-*hth* segregated in a Mendelian fashion.

F_3 plants have both na-pi and β-hth

Northern blot analysis was used to screen F_3 seedlings derived from F_2 line #5.17, four of which are shown in Fig. 2-18B(i). All F_3 plants accumulated Na-PI mRNA and β-HTH mRNA at a level which was approximately the same as in the F_2 line #5.17. The level of Na-PI in F_3 seedlings was estimated, on average, to be 0.5% of the TSP.

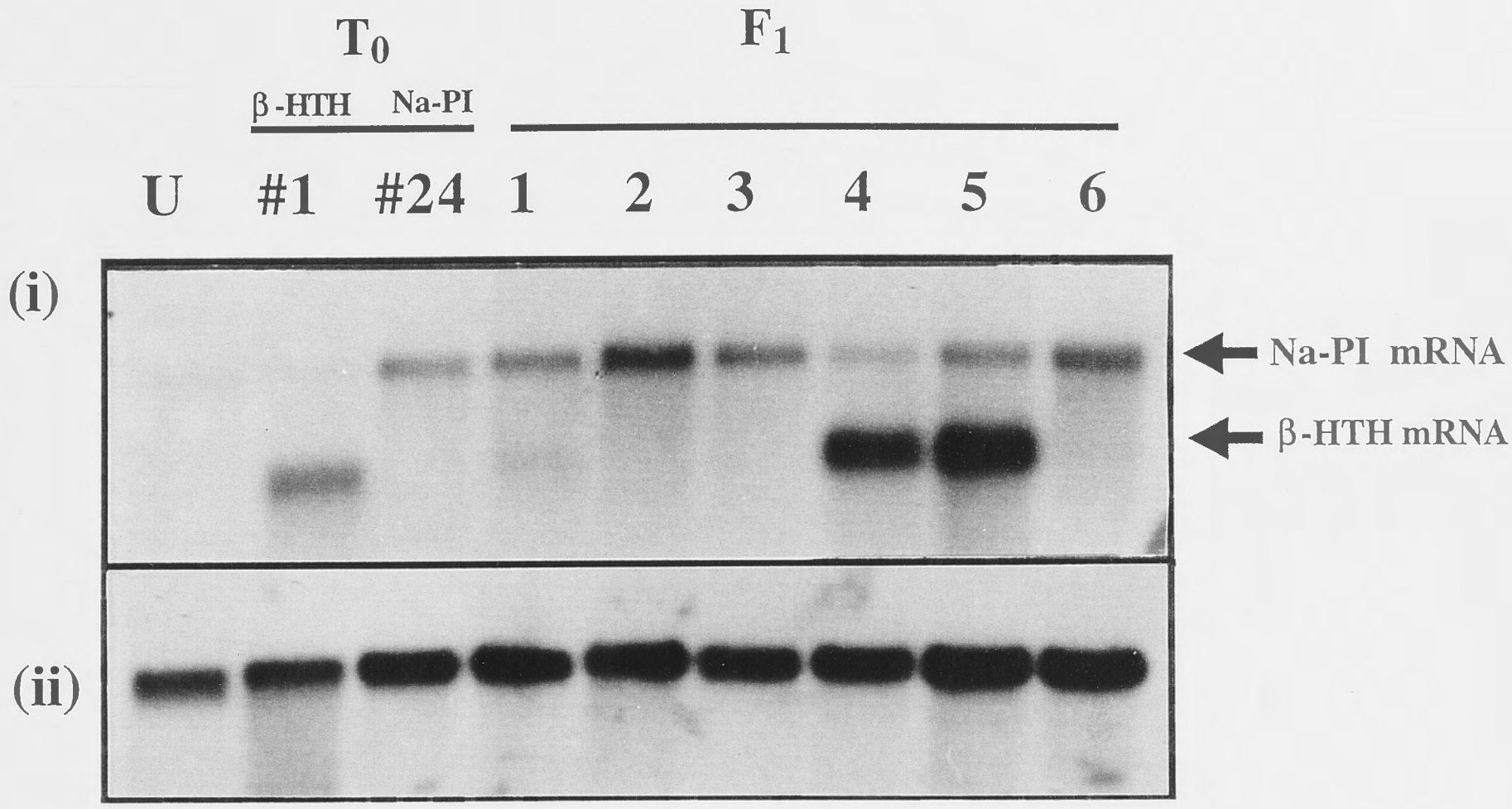
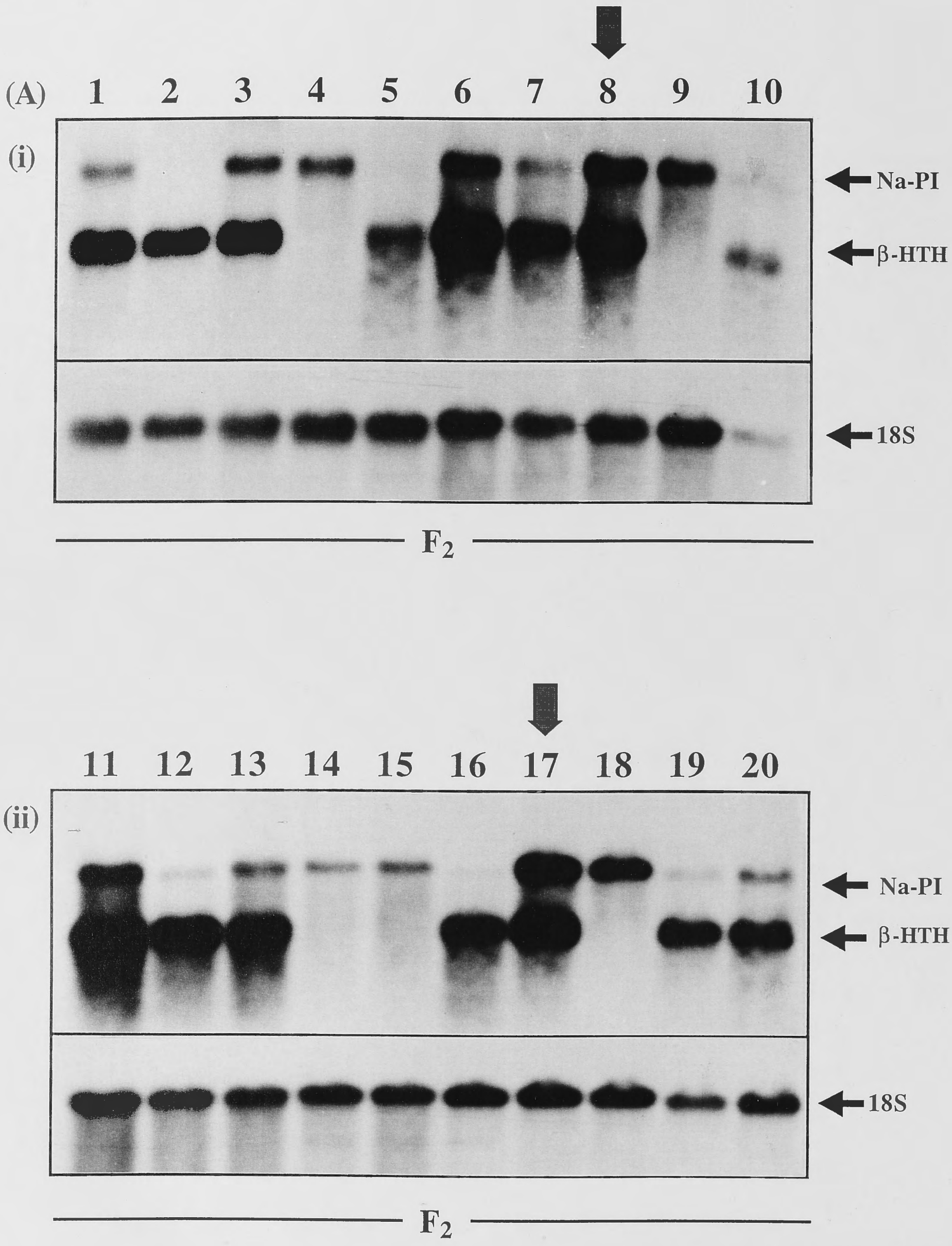


Fig. 2-17 Selection of transgenic tobacco containing both β-HTH and Na-PI, by northern analysis

(i) Fluorograph of a gel containing 5 μg (per track) total RNA from primary transgenic tobacco lines β-HTH-#1 and Na-PI-#24 and F_1 lines (#1-6) and untransformed control (U). β-HTH and Na-PI mRNA were detected by probing with ^{32}P-labelled cDNA. (ii) The same blot was probed with ^{32}P-labelled 18S ribosomal RNA to check for loading. The two F_1 lines #4 and #5 contained both Na-PI and β-HTH mRNA.


(A)
1 2 3 4 5 6 7 8 9 10
(i)
Na-PI
β-HTH
18S
F2
11 12 13 14 15 16 17 18 19 20
(ii)
Na-PI
β-HTH
18S
F2

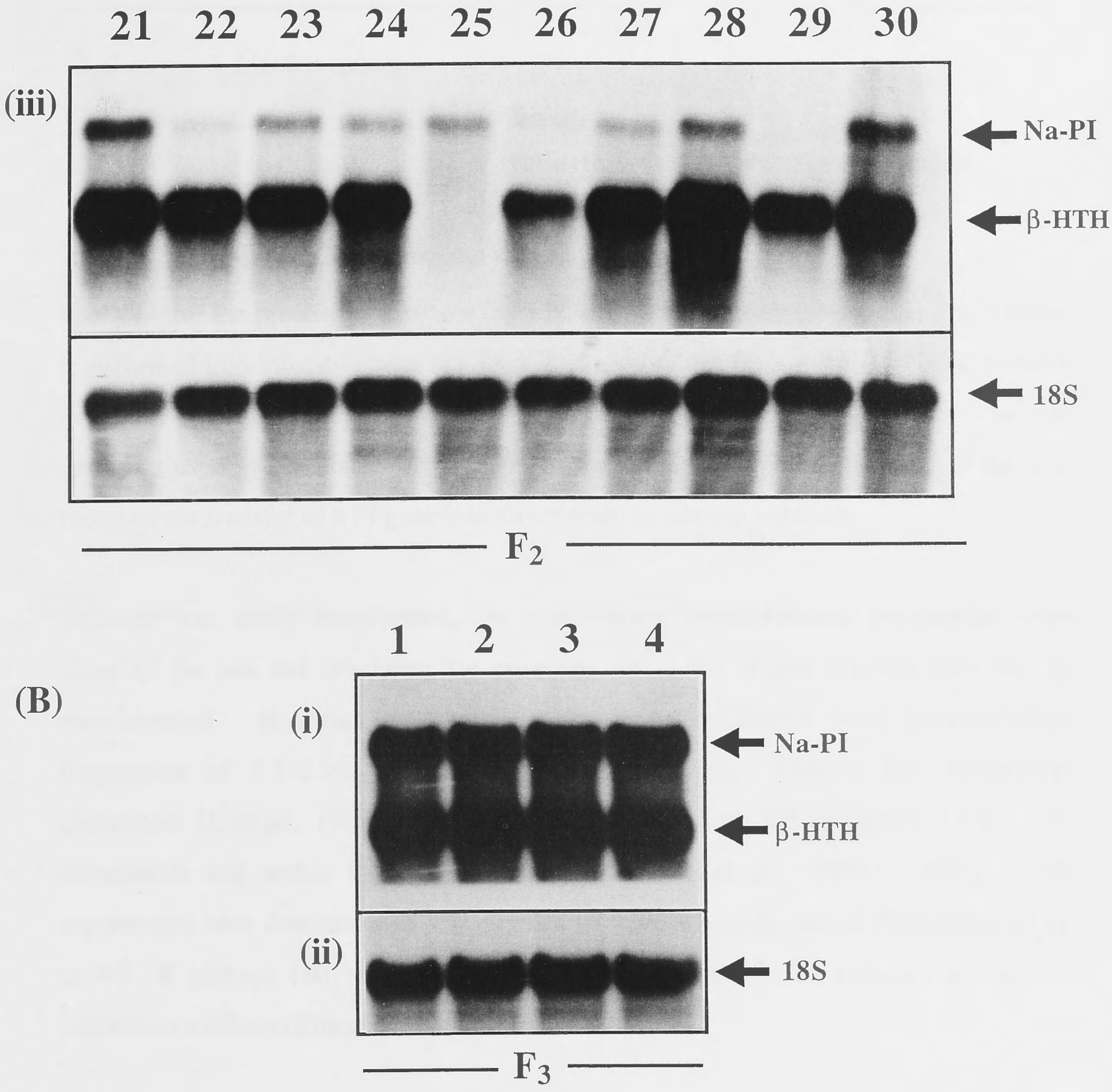


Fig. 2-18 Selection of homozygous F_2 transgenic tobacco containing both β-HTH and Na-PI
(A) Fluorograph of a gels containing 5 μg (per track) total RNA from 30 transgenic tobacco derived from F_1 line #5 (which contains both β-HTH and Na-PI mRNA - see Fig. 2-17). Numbers above the track refer to individual F_2 seedlings. β-HTH and Na-PI mRNA were detected by probing with ^{32}P-labelled cDNA, followed by ^{32}P-labelled 18S ribosomal RNA (underneath). Vertical arrows above line #8 and #17 indicate plants that appear to be homozygous for both genes. (B) (i) Detection of β-HTH and Na-PI mRNA in four F_3 seedlings derived from T_2 line #17. (ii) Same blot in (i) probed with ^{32}P-labelled 18S ribosomal RNA. The *na-pi* and β-*hth* genes segregated as expected (see Table 2-2). F_3 progeny derived from F_2 line #17 contained Na-PI and β-HTH mRNA indicating this line was homozygous for both genes.

2.4 Discussion

2.4.1 Na-PI and β-HTH were detected in transgenic plants

2.4.1.1 Plant transformation

A cDNA for the proteinase inhibitor from *N. alata* or a β-hordothionin from barley was transformed into tobacco under the control of a promoter from a Rubisco small subunit gene. In addition, Na-PI was transferred into pea and subterranean clover. The *bar* gene was used as a selectable marker in all transformation experiments. This is the first report of the transfer of a PI gene into either grain or pasture legumes.

Tobacco was easily transformed, but much lower transformation frequencies were observed for pea and subclover; for example, only 1.2% of pea explants gave rise to transformants. However, this was not significantly different from transformation frequencies of 1.5-2.5% reported for the pea cultivars Rhondo and Greenfeast (Schroeder H. *et al.,* 1993). The transformation efficiency for subclover (1.8%), was comparable and within the range achieved by Khan *et al.,* (1994). More recent experiments have demonstrated it is possible to achieve transformation efficiencies of up to 4%, if glucose (10 mM) and acetosyringone (20 mM) are included in the co-cultivation medium (Khan *et al.,* 1994).

In all three species transformed with *na-pi*, physiological characters such as plant appearance, development time and fertility in the glasshouse remained unaffected by the integration of the *na-pi* gene into the genome.

Despite the possible toxic effects of thionins, transgenic tobacco plants containing β-HTH were phenotypically indistinguishable from untransformed tobacco plants. Fertility was unaffected although some primary transgenic plants were delayed in their development in the glasshouse. This could, however, be attributed to growth in tissue

culture as T_1 and T_2 propagated from seed, in most cases, grew as quickly as untransformed controls.

These observations provided evidence that the presence of either *na-pi* or β-*hth* did not disrupt important physiological traits or modify fundamental plant function. This is an important criterion in assessing the usefulness of a gene in crop plants, as it would be disadvantageous if an inserted gene caused deleterious effects on plant height, yield or development time.

2.4.1.2 Factors governing mRNA variability

mRNA variability within a plant species

The presence of a single mRNA transcript specific for either Na-PI or β-HTH indicated that transcription and stable accumulation occurred in the leaves of transgenic plants. There was up to a 12-fold variation of Na-PI mRNA levels amongst the independent T_0 transformed lines of tobacco, pea and subclover (Figs. 2-4, 2-10 and 2-12) and up to a 3-fold variation of β-HTH mRNA amongst the five tobacco transformants tested (Fig. 2-14).

Variation in levels of transgene products has been observed in nearly all plant systems containing foreign genes (Willmitzer, 1988). The difference in transcript accumulation is not understood, but there are a number of possible ways in which the mRNA levels could vary. Firstly, the level of mRNA may depend on the number of copies of the T-DNA that were integrated into the genome. Zambryski (1988) found that on average, *Agrobacterium* transferred three copies of the T-DNA into various plant species. There must be other factors governing mRNA variability because in most cases there is no gene copy number relationship, and, in fact high gene copy number has been correlated with both high expression levels (Higgins *et al.,* 1988), or lowered expression levels (Hobbs *et al.,* 1990; Linn *et al.,* 1990).

A second reason as to why mRNA accumulation varies in independent transformants, is that the position of gene insertion, in the genome may affect its performance. One

consequence of this so called 'position effect' is that the chromosomal sequences flanking the T-DNA at the insertion site will influence transcription (Jones *et al.,* 1987).

mRNA variability between plant species

As well as detecting Na-PI mRNA variation *within* a single species, there appeared to be variation *between* the three plant species. For the small number of primary transformants analysed here, tobacco contained a higher level of Na-PI mRNA (Fig. 2-4), on average, than the level found in either transgenic pea (Fig. 2-10) or subclover (data not shown). There are a number of reasons why Na-PI accumulation appeared to be the highest in tobacco.

First, the transcription of Na-PI in *N. tabacum* may have been more efficient as the *na-pi* gene originated from the closely related species, *N. alata.* In pea and subclover, both species are unrelated to *N. alata* and belong to an entirely different family. Furthermore, mRNA may have been more stable in tobacco, compared to pea or subclover with the result being greater accumulation of Na-PI mRNA in tobacco.

Another explanation is that the *Arabidopsis* small subunit of Rubisco (ASSU) promoter may perform differently in the three plant species, leading to differences in mRNA accumulation, in a way that has been reported for the Cauliflower Mosaic Virus 35S (CaMV 35S) promoter (Tabe *et al.,* 1995). The mRNA for sunflower albumin (SSA) driven by the CaMV 35S promoter accumulated to high levels in tobacco, yet when the same chimeric gene was transferred to lucerne (alfalfa), the average level of mRNA was less than a quarter of that seen, on average, in tobacco. In this case, higher levels of SSA mRNA were achieved only by replacing CaMV 35S with ASSU (Tabe *et al.,* 1995), providing evidence that a promoter may be preferentially expressed according to the host cell type. This demonstrated a single constitutive promoter may not be suited for high expression of a transgene in every plant species, so perhaps the levels Na-PI mRNA could be further augmented in pea and subclover under the transcriptional control of a constitutive promoter, other than ASSU.

Finally, at least for subclover and pea, it is possible that too few primary transformants were screened, thereby reducing the potential for selecting an individual with high Na-PI mRNA. To substantiate the claim that Na-PI mRNA accumulates to higher levels in tobacco, than in legumes, further work is required. For example, the average level of Na-PI mRNA would have to be estimated from at least 20 transformants of each species.

2.4.1.3 Variability of polypeptide levels and correlation with mRNA.

Detection of Na-PI in transgenic plants

Not surprisingly, the amounts of Na-PI polypeptides detected varied between individual transformants. In T_0 tobacco, the variation in polypeptide level generally correlated with the variation in mRNA levels, for example plant lines #2, #5, #13 and #24 had the highest levels of mRNA and correspondingly high levels of the M_r ~6000 Na-PI polypeptides. However, there appeared to be some exceptions. For instance, lines #4 and #21 contained low levels of Na-PI mRNA yet a higher than expected amount of Na-PI polypeptide. This turned out to be an artefact of sampling leaves of slightly different age.

In the analysis of primary transformants, the vegetative clones of each line used in northern and western analysis were grown at separate times. This complicated the interpretation of these data because environmental factors are now known to come into play. At the time of these analyses, very little was known about the expression of *na-pi* under the control of ASSU, especially in relation to developmental time and light conditions, and the effects that these factors have on Na-PI accumulation. For example, it is now known that the level of Na-PI accumulation decreases with increasing leaf age (Fig. 2-6). It is possible therefore, that the leaves from plants #4 or #21, harvested for western analysis were slightly younger, than those leaves harvested for northern analysis. Once the age-specific expression of Na-PI was recognised, great care was taken in subsequent experiments to harvest leaves of a predetermined age and size from plants of a known developmental age, and where appropriate, the leaf was cut longitudinally into two pieces - half being used for northern analysis and the remaining half for western analysis. Consequently, in all later experiments (that is, other than the analysis of

primary transformants), there was a consistent, positive correlation between mRNA levels and protein levels (data not shown).

The level of Na-PI in T_0 transgenic tobacco, pea and subclover was 0.28%, 0.1% and 0.2%, respectively and was comparable to the levels of potato proteinase inhibitor II (pin2) observed in transgenic tobacco (McManus *et al.,* 1994; Jongsma *et al.,* 1995). However, higher levels of PIs have been attained in transgenic rice. For example, when a pin2 gene was controlled by its own wound-inducible promoter, together with the first intron of the rice actin 1 *(Act1)* gene, the protein accumulated about 2% of total soluble protein in transgenic rice (Duan *et al.,* 1996). Similarly, cowpea trypsin inhibitor, driven by a constitutive promoter from *Act1,* led to high level accumulation (~2.7%) in rice (Xu *et al.,* 1996). In light of these recent findings, it is possible that transcription and, ultimately, the level of Na-PI in transgenic plants could be increased by replacing ASSU with a stronger constitutive promoter, or the double CaMV 35S promoter (Bekkaoui *et al.,* 1990). Alternatively, inserting an enhancer region between the ASSU promoter and the Na-PI cDNA coding region, such as the Kozac-consensus sequence, which is thought to enhance translation (Kozac, 1989), could increase the accumulation of Na-PI in transgenic plants.

Detection of β-HTH in transgenic tobacco

β-HTH peptides not detectable in total protein extracts from the leaves of transgenic tobacco plants, were identified only if the protein extract was enriched for thionins by ethanol precipitation (Fig. 2-14B). Low level accumulation in total protein extracts was also reported by Carmona *et al.,* (1993a; 1993b) and Florack *et al.,* (1994). In the analysis of the four tobacco transformants (Fig. 2-14), it appeared that the β-HTH mRNA level did not always correlate with the level of β-HTH protein. Differences in protein loading were not accounted for and it is possible that western blotting did not give a fair estimate of the protein present. Florack *et al.,* (1994) also commented that the level of α-HTH mRNA was not consistent with the expected level of β-HTH protein in their transgenic tobacco. At this stage, there is not enough evidence to correlate thionin mRNA with thionin protein. Further investigation is needed and the answer

could lie in an extensive study of the level of β-HTH mRNA and protein in at least 20 independent transformants.

It was not possible to estimate the levels of thionin in the transgenic plants, as β-HTH purified from barley endosperm was not available. In other studies, most tobacco plants transformed with α-HTH accumulated peptides to levels of 0.01-0.03% TSP, although plants with α-HTH levels between 0.1% and 0.7% TSP have been produced (Florack *et al.*, 1994). To achieve this higher level of β-HTH, tobacco was transformed with constructs which had been altered in codon usage, in favour of solanaceous crops and all had a modified CaMV 35S promoter containing a doubled enhancer sequence and the *nos* terminator sequence (Florack *et al.*, 1994). Carmona *et al.* (1993b) estimated that their transgenic tobacco accumulated ~0.3% α-HTH of TSP, i.e., within the range observed by Florack *et al.*, (1994). The amount of β-HTH in the leaves of transformed tobacco, produced in this study, needs to be estimated by comparison with a known amount of β-HTH, purified from barley.

2.4.2 Cleavage of the Na-PI and β-HTH precursors

The 40.3 kDa Na-PI precursor was apparently cleaved correctly in leaves of transgenic plants because only polypeptides of the expected size (M_r ~6000) were detected. Heath *et al.*, (1995) showed that there are six repeated linker regions containing aspartate, asparagine, glutamate and lysine residues in the Na-PI precursor which correspond to the sites where the protein is cleaved by an endoproteinase in the stigmas of ornamental tobacco (Fig. 1-1). The protease-sensitive sequence, EEKKND, that links the 6 kDa peptide domains has not been described in any other plant protein precursor. Cleavage in this linker region probably occurs between the asparagine (N) and aspartate (D) residues by an endoproteinase which cleaves on the carboxyl side of asparagine or the amino side of aspartate (Heath *et al.*, 1995). Following the initial cleavage of the Na-PI precursor into five homologous PIs, a non-specific amino- and carboxypeptidase completely removes the five amino acids EEKKN between the C-terminus of one peptide and the N-terminus of the next one (Heath *et al.*, 1995). Processing is not always precise, so an additional amino acid may be removed from the N- and/or C- termini to generate

"ragged ends". In this way, each proteinase inhibitor may be present as up to four truncated isoforms which do not differ in anti-tryptic or anti-chymotryptic activity (Heath *et al.*, 1995).

Cleavage of Na-PI in transgenic plants indicated that leaves have an enzyme which recognised the cleavage site in the Na-PI precursor. In support of this claim, asparagine-specific endoproteinases appear to be widespread in the plant kingdom but most of those so far characterised are from seeds and are predominantly localised in the vacuole (Boller and Wiemken, 1987). One example is the asparagine-specific endoproteinase from soybeans, which is thought to be responsible for cleavage of glycinin (Scott *et al.*, 1992). Another is a vacuolar protease from castor bean seeds which processed three different proproteins on the carboxyl side of an asparagine residue (Hara-Nishimura *et al.*, 1991). Tobacco leaves are also likely to contain asparagine-specific endoproteinases as in the leaves of transgenic plants, foreign proproteins were shown to be cleaved. For example, proteinase inhibitors I and II (originally from tomato leaves) were cleaved in the leaves of transgenic tobacco (Johnson *et al.*, 1989), demonstrating the intergeneric conservation of endoproteinases. These PIs required removal of pre- and pro-sequences from inhibitor (Graham *et al.,* 1985a) and removal of a pre–sequence from inhibitor II (Graham *et al.,* 1985b). Further evidence for endoproteinase activity is that in the leaves of transgenic tobacco, a cysteine proteinase inhibitor from rice seeds was processed (Masoud *et al.*, 1993), the vacuolar targeting signal from barley lectins was post-translationally cleaved (Schroeder M. *et al.*, 1993) and barley thionin precursors were processed (Carmona *et al.,* 1993b; Florack *et al.,* 1994). In the present study, the cleavage of the Na-PI precursor in pea and subclover implies the presence of endoproteinases that are related at an inter-family level.

Although the size of the observed β-HTH peptide was slightly larger (M_r ~ 8500) than that expected (M_r ~ 5000) from the deduced amino acid sequence, the β-HTH is assumed to have been cleaved in the leaves of transgenic tobacco because it co-migrated with β-purothionin purified from wheat. In an equivalent study in transgenic tobacco, the signal and acidic peptides of α-HTH, (which differs from β-HTH, by only 13 amino

acids; Hernández-Lucas *et al.,* 1986) were correctly cleaved to yield the mature thionin, estimated by SDS-PAGE to be 5000 (Carmona *et al.,* 1993b; Florack *et al.,* 1994). Furthermore, a polypeptide of ~7000 in leaf extracts of transgenic tobacco co-migrated with purified wheat β-purothionin, indicating cleavage of wheat β-purothionin in leaves (P. Hughes, pers. comm.). The size of both the purified β-purothionin and the co-migrating protein in leaf extracts, was estimated by comparison to proteins of known molecular weight, and, interestingly, was higher than expected (P. Hughes; pers. comm.). One explanation as to why the M_r of β-HTH or β-PTH in transgenic tobacco plants both in my or P. Hughes' study, respectively, was larger than expected is that highly basic proteins, like thionins, tend to bind more SDS than non-basic proteins. This can significantly decrease their mobility in polyacrylamide gels (Lambin, 1978; Hames and Rickwood, 1990). An alternative, but unlikely explanation is that the acidic peptide was not post-translationally cleaved, however, if this was the true, then the size of the β-HTH observed by western blotting would be approximately M_r 13 000. In addition, without cleavage of the signal and acidic peptides, β-HTH would not be biologically active, yet antimicrobial activity of transgenic tobacco containing β-HTH was demonstrated in fungal and bacterial bioassays (Chapter 4). Thus, assuming β-HTH was cleaved, transgenic tobacco plants must have a mechanism to protect cells from the toxic effects of the mature thionin, as in previous studies, barley leaf thionins were found to damage cultured tobacco mesophyll protoplasts so that callus formation was inhibited (Reimann-Philipp *et al.,* 1989).

2.4.3 Expression of Na-PI as a function of developmental age and tissue type

The level of Na-PI in leaves of increasing age and in different tissues was examined to give an indication of age-specific or tissue-specific accumulation of Na-PI in transgenic tobacco. Na-PI peptides were maximal in the youngest leaves, and declined steadily to much lower levels in senescing leaves on a total protein basis (Fig. 2-6). It would be interesting to determine if it is the down-regulation of the promoter which causes a decrease in Na-PI as tissues age, by estimating the level of transcript in leaves of different ages. In an analogous study, pea seed vicilin (per unit soluble protein) was also

higher in younger leaves than in the older leaves. This was thought to be either a function of the CaMV 35S enhancer region, which was fused to the vicilin promoter or due to increasing proteolytic activity with leaf age (Higgins and Spencer, 1991).

The organ specificity of ASSU-Na-PI in transgenic tobacco was determined by western blotting and calculated on a fresh weight basis (Fig. 2-7). Young and mature leaves contained the most Na-PI (26-60 ng/gm); stems, petioles, sepals, pods and petals contained moderate levels (6-13 ng/gm); while Na-PI was undetected in seeds. The tissue-specificity of ASSU has been studied previously using the *nptII* reporter gene encoding neomycin phosphotransferase II (De Almeida *et al.,* 1989). The *nptII* chimeric gene driven by the *Ats1A* (or ASSU) promoter was expressed abundantly in all green organs, with lesser but still significant amounts observed in petals and roots, and these results are consistent with the findings presented here.

Proteins with Na-PI specificity were also detected in the sepals, pods and petals of untransformed plants (Fig. 2-7). It is not surprising that tissues of *N. tabacum* contain peptides which cross-react with the *N. alata* antibody, as it was recently shown that wounded tobacco leaves contain small ~6 kDa peptides which have sequence similarity to Na-PI (Pearce *et al.,* 1993). Furthermore, at the mRNA level, Atkinson *et al.,* (1993a) showed that RNA isolated from the styles of tobacco contained a 1.4 kb mRNA species which hybridised to the Na-PI cDNA from *N. alata* stigmas and flowers.

2.4.4 Stable inheritance of Na-PI and β-HTH

The finding that the β-*hth* (or *na-pi*) and *bar* genes integrated into the tobacco, (or pea and subclover) genomes at single loci enabled T_1 plants to be screened, simply by their tolerance to PPT. T_2 plant lines homozygous for Na-PI and β-HTH were sought not only to establish if these genes were stably inherited in the germline, but to enable a homogenous population of transgenic plants to be assessed in insect, fungal and bacterial bioassays. This goal was achieved with the finding that T_2 progeny derived from tobacco line #24.2, (or β-HTH line #1.3), pea line #10.4, and subclover lines #4 and #6 were homozygous for *bar* and *na-pi* (or β-*hth).*

2.4.5 F_3 progeny were homozygous for two defence genes

All F_3 seedlings derived from plant line #5.17 had equal amounts of Na-PI mRNA and β-HTH mRNA and were resistant to PPT, which, together indicated that this line was homozygous for both genes. This work describes the first report of transgenic tobacco containing both a proteinase inhibitor gene and a thionin gene. There are, however, concurrent reports of more than one defence related gene in other transgenic plants. For example, chitinases and β-1,3-glucanases have been transformed into tomato (Jonedijk *et al.,* 1995), and tobacco and sweet potatoes have been transformed with the cowpea trypsin inhibitor and snowdrop lectin, (Boulter *et al.,* 1990; Newell *et al.,* 1995, respectively). In all cases plants were transformed with a single plasmid containing both genes. In comparison, the highest expressing Na-PI and β-HTH plants were cross fertilised to produce transgenic tobacco containing both defence genes.

2.5 Chapter summary

cDNA clones encoding the precursors of a proteinase inhibitor from *N. alata* (Na-PI) and a thionin from barley (β-HTH) were reconstructed for expression in leaves and transferred into tobacco. Na-PI or β-HTH mRNA accumulated stably in T_0 tobacco leaves. In addition, the precursors for Na-PI and β-HTH were cleaved in the leaves of transgenic tobacco to yield M_r ~6000 or M_r ~8 500 polypeptides, respectively. This indicated removal of the signal peptide and post-translational cleavage to form the mature Na-PI or β-HTH.

Na-PI accumulation decreased with leaf maturity and was upregulated only in the green tissues of transgenic tobacco, with maximum levels in leaves. Preliminary studies indicated that Na-PI was not secreted from the cell, as it was not detected extracellularly. The Na-PI cDNA was also transferred to pea and subclover. M_r ~6000 polypeptides accumulated to levels of 0.1% and 0.2%, respectively, of the soluble protein, indicating cleavage of the Na-PI precursor in legume species. The *na-pi* and β-*hth* genes segregated as dominant Mendelian traits and were stably transmitted for at least two

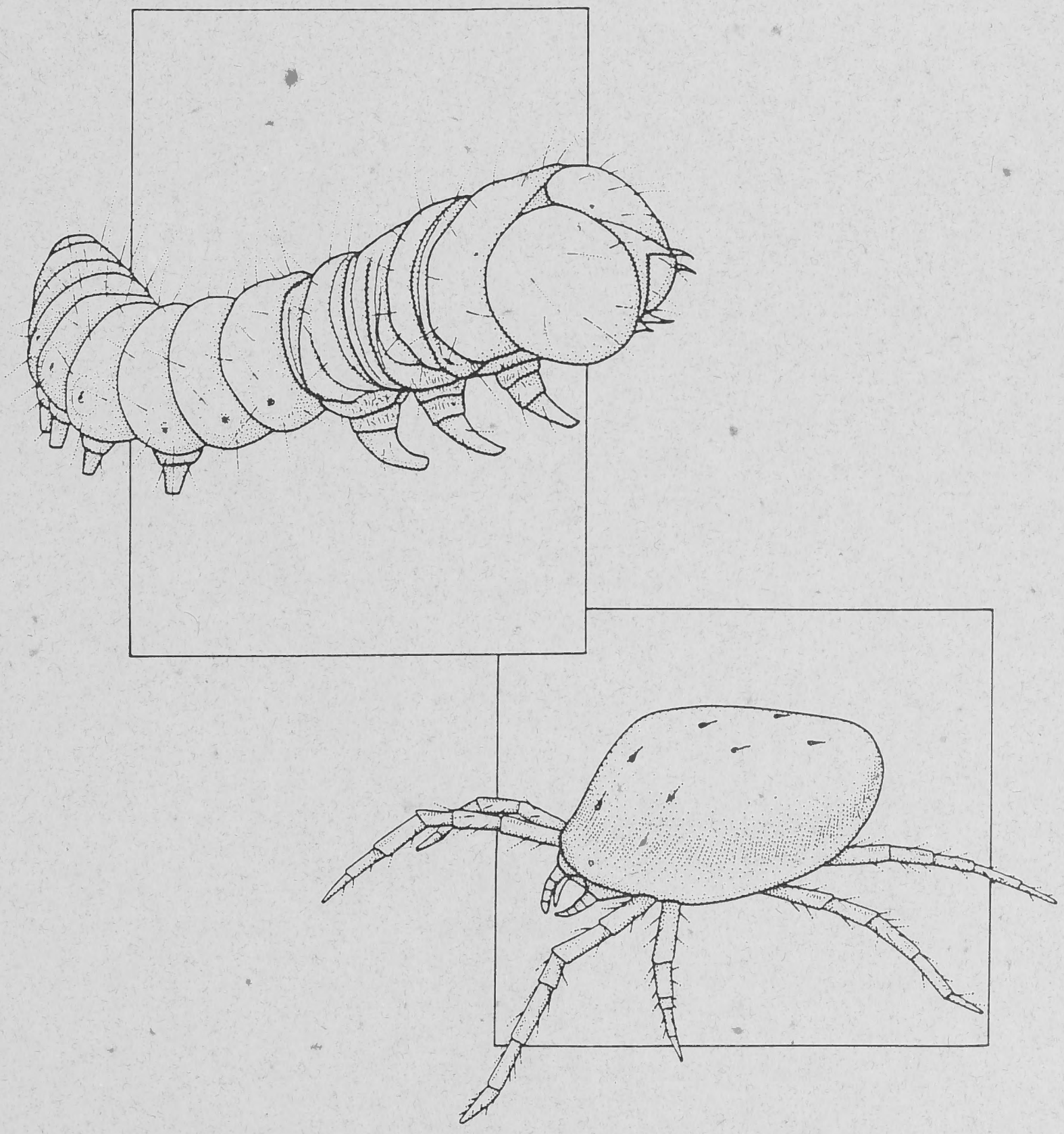
generations of all three species (in the case of Na-PI), or in tobacco, (in the case of β-HTH).

Tobacco containing the highest amount of each foreign peptide were cross fertilised to produce lines containing both genes. Such F_1 individuals were propagated to the next generation, allowing the selection of an F_2 individual, homozygous for both Na-PI and β-HTH. Homozygosity of this selected individual was confirmed by showing that F_3 progeny derived from this line contained both genes.

The next chapter describes the performance of these transgenic plants in insect bioassays. The aim was to assess whether Na-PI and/or β-HTH confer enhanced pest resistance to plants.

Chapter Three

Insect Bioassays



Chapter Three

The insecticidal properties of plants containing genes with potential for plant defence

3.1 Introduction

Helicoverpa species are major pests of crops in Australia including peas, cotton, coarse grains, oil seeds and many vegetables (Fig. 3-1A, B and C). Currently the major control of *Helicoverpa* species is through multiple applications of insecticides and there has been little success in identifying plant varieties with natural resistance to these pests (van Emden *et al.,* 1988). Consequently, there has been great interest in controlling *Helicoverpa* using biotechnological approaches.

Evidence that Na-PI has potential to offer protection to transgenic plants from pests has come from the finding that the chymotrypsin and trypsin inhibitors derived from the Na-PI precursor could inhibit the proteolytic activity of gut extracts from a range of insects, *in vitro* (Heath, 1994, Heath *et al.,* 1997). As Na-PI significantly inhibited the serine proteases of *Helicoverpa armigera* and *H. punctigera,* these species were chosen for further study using artificial diets. The biomass of *H. punctigera* larvae fed on artificial diets containing 0.26% (w/w) Na-PI was reduced by 54%, compared to larvae fed a control diet (Heath, 1994; Heath *et al.,* 1997). Given that Na-PI was effective in reducing larval weight at a level of protein which is achievable in transgenic plants, the next logical step was to create transgenic plants containing Na-PI (described in Chapter Two) and assess them for increased resistance to insects and arthropods.

It is known that thionins are toxic to *Manduca sexta* (tobacco hornworm), if injected directly into the hemocoel (Kramer *et al.,* 1979). Although the current focus for thionin

research is primarily on their antifungal and antimicrobial properties (see Chapter Four), I decided to investigate whether thionins have insecticidal activity by testing the tolerance of transgenic tobacco containing β-HTH to attack by *H. armigera.*

Transgenic plants which contain more than one putative defence gene may be more protected from damage by insects. For example, the effect of pea lectin and cowpea trypsin inhibitor were additive in protecting transgenic tobacco from *H. virescens* (Boulter *et al.,* 1990). One *in vitro* study showed that a barley trypsin inhibitor together with a wheat purothionin were synergistic in their inhibitory effect on the fungus *Fusarium culmorum.* It is plausible, therefore, that a combination of a PI and a thionin may also interact synergistically on insects. These precedents provided good reason to use *H. armigera* bioassays to determine if transgenic tobacco which contained both Na-PI and β-HTH were more resistant to *H. armigera* than transgenic plants which contained only one gene.

Redlegged earth mite (RLEM), *Halotydeus destructor* (Tucker) (Acarina: Penthaleidae) is a major pest of pasture legumes, and is widespread throughout Southern Australia. RLEM (Fig. 3-1D, E and F) feed predominantly on broad-leaved plants and grasses, including subterranean clover, canola, white clover, young wheat, tobacco and pea. As a result of RLEM feeding, leaves of infested plants are characterised by silver blemishes (Fig. 3-1E), which lead to wilting and growth reduction.

The antimetabolic effects of a range of proteins against RLEM had previously been tested in artificial diets (James Ridsdill-Smith; unpublished). Overall, there were slight responses to Na-PI, pea lectin and wheat germ lectin. For example, when Na-PI was included in artificial diets at levels of 100 µg/ml, the number of progeny was reduced by 24% in four experiments, however, in the fifth experiment, there was an increase in the number of progeny (James Ridsdill-Smith; pers. comm.). The effect of Na-PI on RLEM reproduction, although variable was sufficiently promising to lead me to question whether Na-PI could protect transgenic subclover from RLEM. My role in this work

involved the transfer of a cDNA encoding the gene for Na-PI into subclover (see Chapter Two). I characterised selected lines and provided confirmation they were homozygous. High expressing T_2 lines were sent to CSIRO, Division of Entomology, Perth for testing by James Ridsdill-Smith.

My aims were to investigate the tolerance of (a) transgenic tobacco and pea containing Na-PI to *H. armigera* and *H. punctigera* and (b) transgenic tobacco containing β-HTH alone and in combination with Na-PI to *H. armigera* feeding. I also report preliminary findings with transgenic subclover containing Na-PI, which were aimed at testing their effectiveness against RLEM.

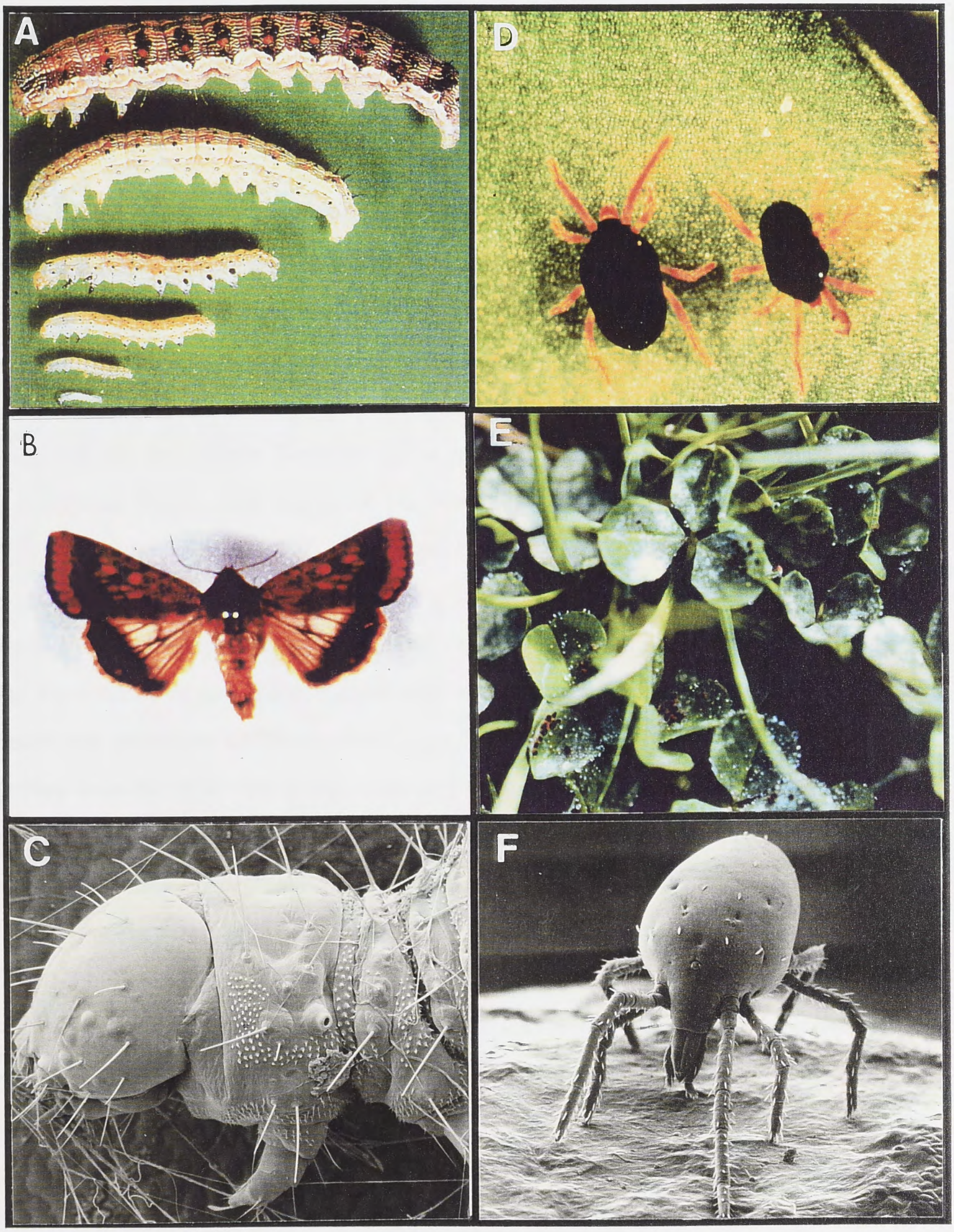


Fig. 3-1. Pests of tobacco, peas and subterranean clover
A. *Helicoverpa armigera* larvae at different stages of development. The larva at the bottom of the photograph is newly hatched (neonate), while the largest is about 14 days old. *H. punctigera* larvae are physiologically identical. (From Lamb *et al.*, 1987). B. *H. armigera* moth. C. Scanning electronmicrograph (SEM) of neonate *H. armigera* larva. D. Redlegged earth mites. E. RLEM feeding on subclover leaves which show "silvering" and extent of leaf damage. F. SEM of redlegged earth mite (from Craig and Beaton, 1996).

3.2 Materials and Methods

3.2.1 Feeding trials with pests of tobacco and pea

3.2.1.1 *Helicoverpa armigera* feeding trial

Neonates of *H. armigera* (Hübner) were reared from a colony originally collected from Narrabri, NSW in 1993 and maintained as a general rearing strain on a high protein and wheat diet at 25°C and 40-50% relative humidity. Unfed neonates were used in feeding trials within 24 h of hatching. The initial weight of larvae was estimated from the mean weight of 100 individuals. Neonate larvae were fed on leaves from either untransformed or transgenic plants. The transgenic plants are fully described in Chapter Two. Primary transgenic (lines #13 and #24) and untransformed (W38) tobacco plants were clonally propagated in tissue culture, transferred to soil and grown in a greenhouse with a 24°C day /12°C night temperature regime. Tobacco leaves, 20 cm in length, were excised, the leaf veins removed and the remaining portion was cut into approximately 2 cm x 3 cm pieces and placed on 0.8% w/v water agar in 9 cm petri-dishes. For pea, T_1 seedlings derived from line #10 were grown, screened for tolerance to PPT to select the transgenic plants from this segregating population. Control pea seedlings were grown from seeds of untransformed 'Greenfeast' plants. Leaves from PPT-tolerant or untransformed pea plants were prepared as above, except that whole fully expanded leaves were used. On days 0-8 of the bioassay, two tobacco leaf pieces or two whole pea leaves were placed in each petri-dish and thereafter five leaf pieces or leaves were used. One larva was placed in each dish and there were 40 replicates of each treatment. Petri dishes were incubated in humid sealed boxes at 25°C. Larvae were transferred to new dishes containing fresh leaf pieces every day. Living larvae were weighed individually after four days and thereafter every two days to estimate growth rate. Dead larvae were counted daily. The percent pupation was based on the total number of pupae recovered from each treatment with respect to the number of larvae that survived. The percent emergence was the number of larvae which successfully became moths, with respect to the number of pupae.

Each treatment initially had 40 larvae, but, because survival varied between the two treatments, residual maximum likelihood analysis (REML) (Thompson, 1980) was employed for analysis of variance. A comparison of growth rates in the different treatments was made using the GENSTAT® statistical package (Lawes Agricultural Trust, Rothamsted Experimental Station). In all experiments, significance of a difference between means was accepted at $P < 0.05$.

3.2.1.2 *Helicoverpa punctigera* feeding trial

The bioassay was performed as described in section 3.2.1.1 except that second instar larvae instead of neonates were used to minimise larval mortality. The larvae of *H. punctigera* were not as robust as *H. armigera* under the bioassay conditions. For pea, T_2 seedlings derived from lines #10.2 and #10.4 were used in the bioassay and larvae were not taken through to pupation.

3.2.1.3 *Helicoverpa armigera* feeding trial with F_3 transgenic tobacco containing both Na-PI and β-HTH.

The feeding trial to test for insect development on tobacco transformed with either β-HTH alone or both Na-PI and β-HTH against *H. armigera* was completed as described in section 3.2.1.1, except that the plant material differed. T_2 transgenic plants containing a single defence gene were grown from T_1 plants with the highest amount of foreign protein. For example, for Na-PI, T_2 progeny derived from the Na-PI expressing line #24.2 were used while for β-HTH, the bioassay used T_2 progeny derived from β-HTH expressing line #1.3. Transgenic plants which expressed both Na-PI and β-HTH were F_3 progeny from line #5.17. Seeds from each parent line were sown in soil and incubated in a demister for three weeks. After six weeks, each plant was tested using the PPT leaf painting test (section 2.2.6). The herbicide tolerant plants were assayed for the presence mRNA from the two genes by northern blotting (section 2.2.4). Leaves from eight plants of each line were used in the bioassays.

3.2.2 Feeding trial with redlegged earth mite

I provided transgenic subclover plants for these experiments which were performed by James Ridsdill-Smith at CSIRO, Division of Entomology, in Perth.

100 seeds from T_1 subterranean clover from each of the transgenic lines #3.2, #4.1 and #6.1 and an untransformed control were sown into 13 cm pots filled with soil and fertiliser. Pots were watered twice each week with deionised water. Saucers were emptied after 10 min. to avoid overwatering. After three weeks, plants were thinned to six per pot.

100 field-collected deutonymphs (or tritonymphs) were placed in plastic vials containing a damp sponge. In the glasshouse, one vial was emptied over each pot and immediately enclosed using a clear plastic container with fine mesh panels. At the same time each week, for five weeks, the number of mites feeding on leaves was counted. At the completion of one generation (usually five weeks), the mites were aspirated off each plant using a compressed air aspirator into vials containing 70% ethanol. The mites were counted and examined to determine their developmental stage.

Plants were cut off at soil level and scored for leaf damage on a scale of 1-10, where 1 was approximately 10% damage, 8 was all the leaf surface silvered and a score of 10 indicated the leaf was dead. A microscope was used to assess the number and location of mite eggs.

The surface of the soil remaining in the pot was checked for mites, and any that remained were put into a second vial of 70% ethanol.

3.3 Results

3.3.1 Feeding trials with pests of tobacco and pea

3.3.1.1 Assessment of *Helicoverpa armigera* on transgenic plants containing Na-PI

T_0 transgenic tobacco lines #13 and #24 were tested for improved resistance using *H. armigera* because these lines were the highest expressers. For example, leaves of these plants accumulated 0.17% and 0.28% Na-PI of the soluble protein, respectively. For peas, T_0 line #10 contained a detectable level of Na-PI and seeds from this plant were germinated. PPT-tolerant, T_1 seedlings derived from plant #10 were used and leaves from these plants accumulated about 0.07% Na-PI.

Larval mortality

Larval mortality was increased in *H. armigera* populations growing on transgenic tobacco or pea leaves expressing Na-PI (Fig. 3-2A and C). Mortality increased rapidly in the first 8 days on both control and transgenic diets, but this increase was greater for those larvae fed transgenic leaves, as indicated by the relative steepness of the two curves. For transgenic tobacco, larval mortality was high for populations fed leaves from both line #13 and line #24. There did not appear to be any significant difference in number of deaths between the two transgenic lines, even though line #24 accumulated more Na-PI.

After 8 days, the rate the larvae died began to plateau but the difference in the final percentage of insects which died was maintained. At the time of pupation, in an average of three experiments, 63% of larvae on transformed tobacco had died, compared to only 28% of larvae on untransformed tobacco leaves (Table 3-1). In experiments I and III, mortality of larvae fed transgenic leaves was nearly 4-fold higher, relative to control populations. In the second experiment, however, larval mortality was high, even in the population on control leaves and the difference between the populations on control and transgenic diets was about 1.5-fold. The discrepancy was attributed to variation in the

laboratory-reared strains of *H. armigera,* as for each of the three experiments, a fresh population of larvae were used. On average, there was about a 2.3-fold increase in mortality of larvae fed on transgenic tobacco leaves, compared to larvae fed on control tobacco leaves.

Table 3-1. Mortality of *H. armigera* on T_0 transgenic tobacco (line #24) containing Na-PI

Experiment	Control	Transgenic
I	12.5%	47.5%
II	55%	83.3%
III	16%	57%
AVERAGE	**28%**	**63%**

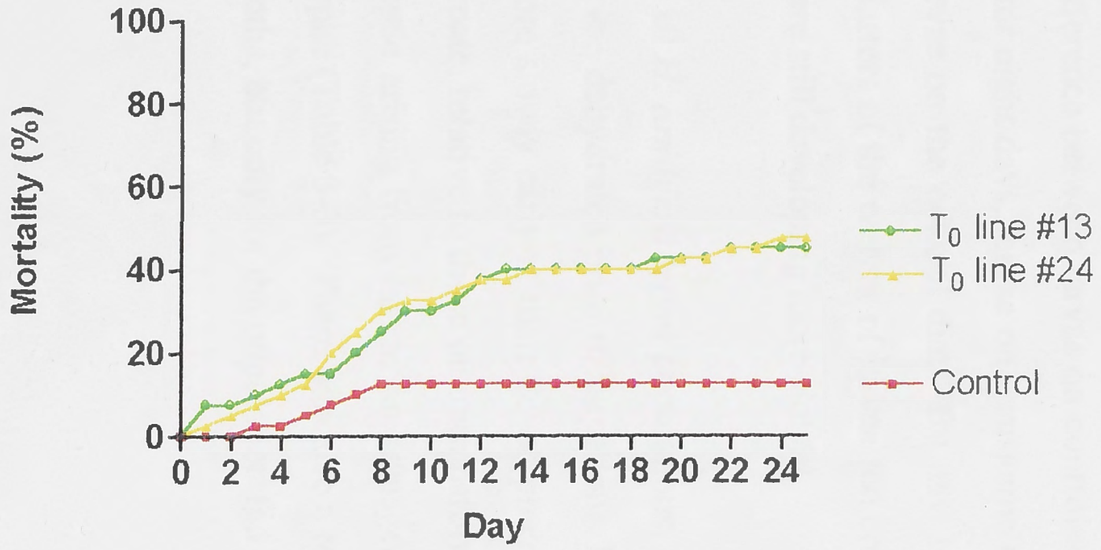
In the case of peas, 53% of the larvae on transgenic leaves died, while the mortality of those feeding on untransformed leaves was 30% (Fig. 3-2C).

Growth rate and development of surviving larvae

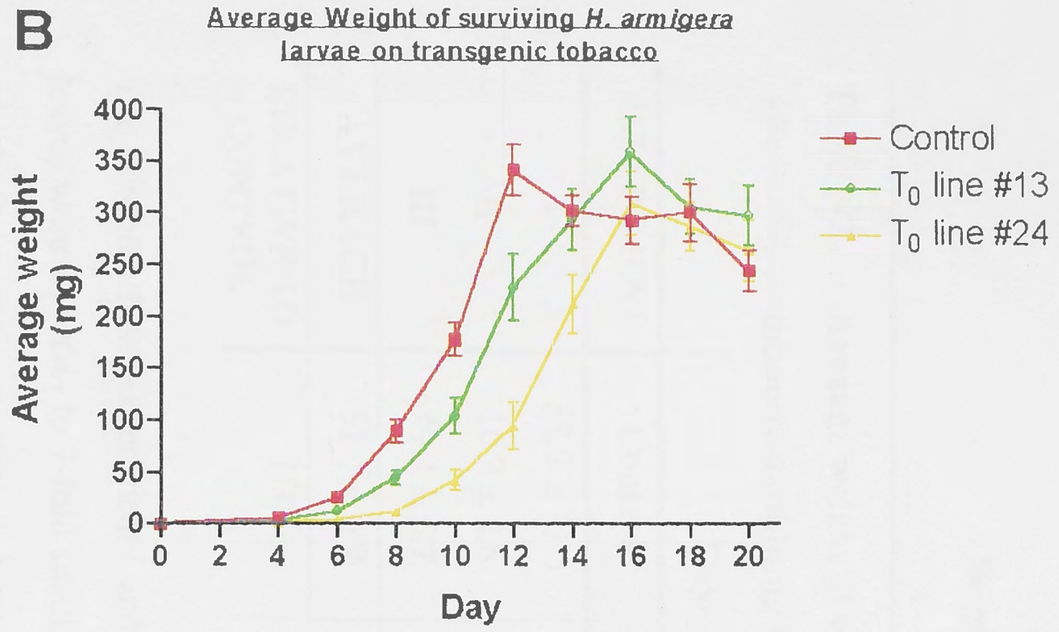
H. armigera growing on transgenic tobacco leaves (lines #13 and #24) were significantly smaller between days 0-12, ($P < 0.001$) compared to larvae fed control leaves (Figs. 3-2B and 3-3). In addition, consumption of transgenic tobacco leaves significantly lowered the rate at which larvae accumulated biomass between days 0-8 ($P < 0.001$) and days 14-18 ($P < 0.001$), compared to insects growing on untransformed leaves. Although there was no effect on the final biomass of the surviving larvae, they required additional time to mature. For example, it took larvae fed control tobacco leaves 8 days to reach 90mg in weight, whereas larvae fed on transgenic tobacco line #13 or line #24 containing Na-PI, required 9.7 or 11.8 days to reach 90 mg, respectively (Fig. 3-2B). It appeared, therefore that the effect of Na-PI on slowing development was dose-dependant, because plant line #24 which accumulated 0.28% Na-PI, was more effective in slowing larval development than line #13, which accumulated 0.17% Na-PI. In an average of three experiments it was shown that *H. armigera* developed more slowly on transgenic tobacco (Table 3-2).

Fig. 3.2 Mortality and growth rate of *Helicoverpa armigera* growing on transgenic tobacco or peas
Data from experiment I are presented. A. Comparison of mortality of *H. armigera* larvae on leaves from control or transgenic tobacco, during a 24 day time period. Forty larvae were applied as neonates at day 0. B. Average weight of *H. armigera* fed leaves from transgenic or control plants. Vertical bars represent standard error of the mean (SEM). C. As for A, but *H. armigera* were grown on leaves from control or transgenic peas. D. Average weight of surviving *H. armigera* on control or transgenic pea leaves. Vertical bars are the SEM. Overall, for *H. armigera* which had ingested leaves from transgenic plants containing Na-PI, larval mortality increased and surviving larvae developed more slowly.

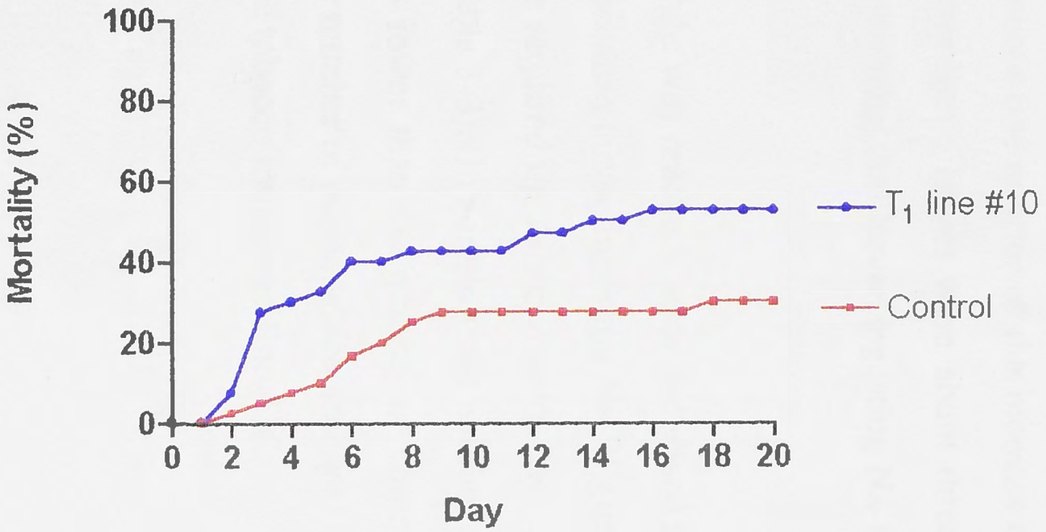
A


Mortality of H. armigera larvae growing on transgenic tobacco
Mortality (%)
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Day
T0 line #13
T0 line #24
Control

B


Average Weight of surviving H. armigera larvae on transgenic tobacco
Average weight (mg)
400
350
300
250
200
150
100
50
0
0 2 4 6 8 10 12 14 16 18 20
Day
Control
T0 line #13
T0 line #24

C


Mortality of H. armigera larvae on transgenic peas
Mortality (%)
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18 20
Day
T1 line #10
Control

D

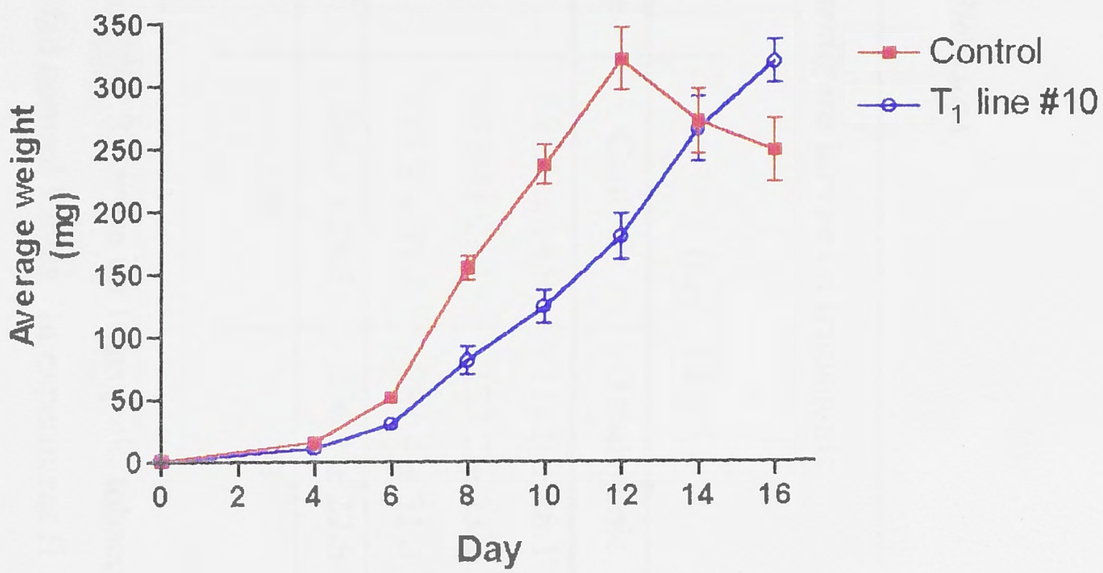

Average weight of surviving H. armigera larvae on transgenic pea
Average weight (mg)
350
300
250
200
150
100
50
0
0 2 4 6 8 10 12 14 16
Day
Control
T1 line #10

Table 3-2. Average weight of surviving *H. armigera* larvae on transgenic tobacco containing Na-PI

	Day 8		Day 14	
EXPERIMENT	**Control**	**Transgenic**	**Control**	**Transgenic**
I	88.8 ± 11.3	11.9 ± 2.2	301.4 ± 14.9	211.9 ± 28.1
II	16.2 ± 2.8	12.0 ± 3.5	193.4± 28.1	123.2 ± 23.2
III	49.4 ± 6.7	13.2 ± 4.4	375.9 ± 33.4	281.4 ± 31.3
AVERAGE	**51.5 ± 6.9**	**12.4 ± 3.4**	**296.3 ±25.5**	**206.1 ± 27.5**
RELATIVE TO CONTROL	**100**	**24**	**100**	**71**

The outcome from experiment I and III was that on day 8, larvae on transgenic tobacco leaves were about 4- to 7-fold smaller than larvae fed control leaves. In experiment II in which there was an overall high mortality and generally poor larval growth, the difference between larvae on control and transgenic diets was not as great. On average, after eight days, larvae on transgenic tobacco were about one quarter of the biomass of larvae on the control diet. By day 14, larvae fed transgenic leaves were about three-quarters of the weight of larvae fed control leaves, indicating that larvae ingesting Na-PI were still developing more slowly.

In all *H. armigera* larval populations, after peak weight was reached, mass decreased as larvae dehydrated prior to pupation. Furthermore, because larvae ingesting Na-PI grew more slowly early in their development, these larvae required up to three extra days to pupate, relative to those on untransformed leaves (Table 3-3). In addition, the number of pupae arising from larvae on transgenic plants was lower than the number of control pupae (Table 3-3). There was also a reduction in the number of pupae which emerged as moths, but only for the population fed leaves from the tobacco transgenic line #24.

Table 3-3. Pupation and pupal emergence of *H. armigera* larvae on transgenic tobacco containing Na-PI

Line	Days to pupation (median)	Percent Pupation*	Emergence¤ (%)
Control tobacco	16	91.4%	100%
Transgenic tobacco #13	17	52.4%	100%
Transgenic tobacco #24	19	54.5%	91%

*Percentage of surviving larvae which pupated. ¤Percentage of pupae that emerged as adults.

Finally, I noted that surviving larvae on the transgenic tobacco diet were more lethargic than those on the control diet.

Fig. 3-3. *H. armigera* larvae feeding on tobacco leaves.
Larvae were photographed at day 10 of the experiment. On the right are larvae growing on control leaves and on the left are larvae on leaves from transgenic tobacco. The amount of leaf tissue is not proportional to total leaf consumption. *H. armigera* larvae grown on leaves from transgenic plants were generally smaller and developed more slowly than larvae on control leaves.

Similarly, in pea, the surviving larvae grown on transgenic leaves accumulated biomass more slowly (Fig. 3-2D), although the difference in mass between larvae grown on control and transgenic leaves was not as marked as those on tobacco. It took ~7 days

for larvae on untransformed pea leaves to weigh 100 mg compared to 9 days for those on transgenic pea leaves. Surviving larvae grown on transgenic pea leaves required an extra two days to pupate (Table 3-4), indicating that these larvae developed more slowly throughout the entire experiment.

Table 3-4. Pupation and pupal emergence of *H. armigera* larval growing on transgenic pea containing Na-PI

Line	Days to pupation (median)	Percent Pupation*	Emergence¤ (%)
Control pea	16	100%	100%
Transgenic pea #10 (T_1)	18	86%	100%

3.3.1.2 Assessment of *Helicoverpa punctigera* on transgenic plants containing Na-PI

T_0 transgenic tobacco line #24 was selected and clonally propagated for testing against *H. punctigera* because this line proved more effective than line #13, in increasing mortality and slowing development of *H. armigera* larvae (see 3.3.1.1). For peas, PPT-tolerant, T_2 seedlings derived from plant #10.2 and #10.4 were used and leaves from these plants accumulated about 0.07% or 0.12% Na-PI, respectively. The data presented for tobacco and pea bioassays using *H. punctigera* represent the results of one experiment. The availability of transgenic leaf material limited the continuation of the tobacco and pea experiments beyond 28 days or 11 days, respectively.

Larval mortality

Larval mortality was significantly higher for *H. punctigera* growing on transgenic tobacco or pea leaves expressing Na-PI, than for those larvae on control leaves (Fig. 3-4A and C). As observed for *H. armigera,* mortality was highest during early development (up to 6 days), in populations on both control and transgenic diets, although those larvae on transgenic leaves were significantly more affected during this period. Mortality of *H. punctigera* reached a plateau at about six days, and in general,

after this time few larvae died (Fig. 3-4A and C). After 28 days, 90% of larvae on transformed tobacco had died, compared to 27.5% of larvae on untransformed tobacco leaves (Fig. 3-4A).

For peas, 60% of the larvae on transgenic leaves from line #10.2 died compared with 70% mortality for line #10.4 while the mortality of those feeding on untransformed pea leaves was 25% (Fig. 3-4C).

Growth rate and development of surviving larvae

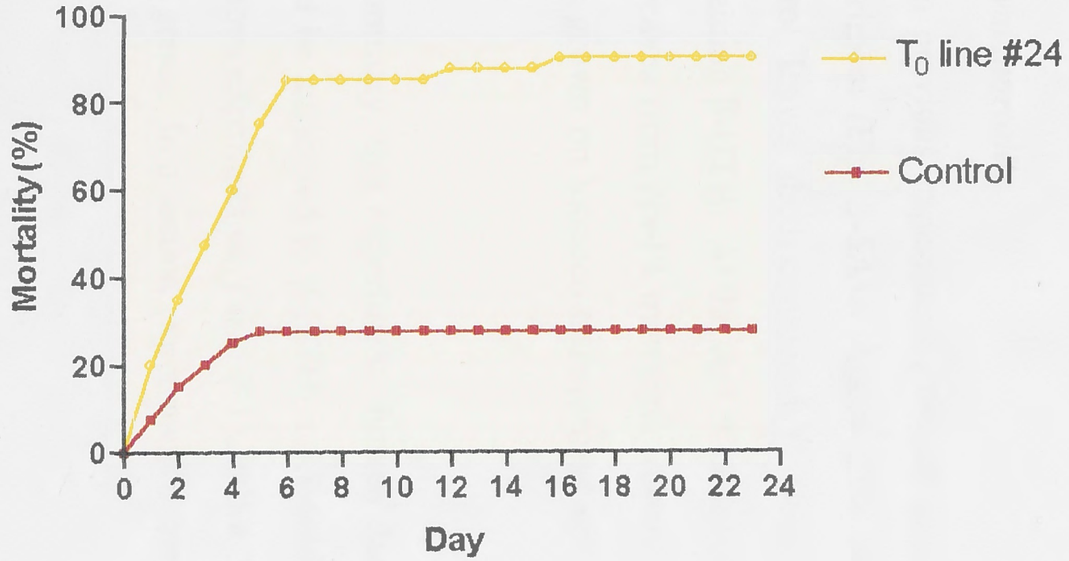
Throughout the experiment, *H. punctigera* fed on transgenic tobacco leaves weighed significantly less ($P < 0.001$) and developed more slowly, compared to larvae fed control leaves (Fig. 3-4B). Growth rate was significantly slower ($P < 0.001$) for larvae grown on transgenic leaves, during the first 20 days. *H. punctigera* on transgenic tobacco leaves were at least seven days behind in development, compared to larvae on control leaves. For example, at 12 days, larvae fed control tobacco leaves weighed about 50 mg, whereas larvae on transgenic tobacco leaves containing Na-PI required 19.8 days to reach this weight. After 16 days, both populations of larvae gained weight at the same rate (i.e. slopes do not differ).

When *H. punctigera* were grown on transgenic pea leaves, the larvae were smaller at each time point and accumulated biomass more slowly, particularly in the first five days (Fig. 3-4D). Overall, the larval population on transgenic leaves experienced a two to four day delay in development. For example, it took 4.7 days for larvae on untransformed pea leaves to reach a weight of 20 mg compared to 7 and 9 days for insects on transgenic pea leaves (lines #10.2 and #10.4, respectively). Therefore, line #10.4 appeared more effective in contributing to a longer developmental delay and this is consistent with the level of Na-PI which accumulates in the leaves of this transgenic plant.

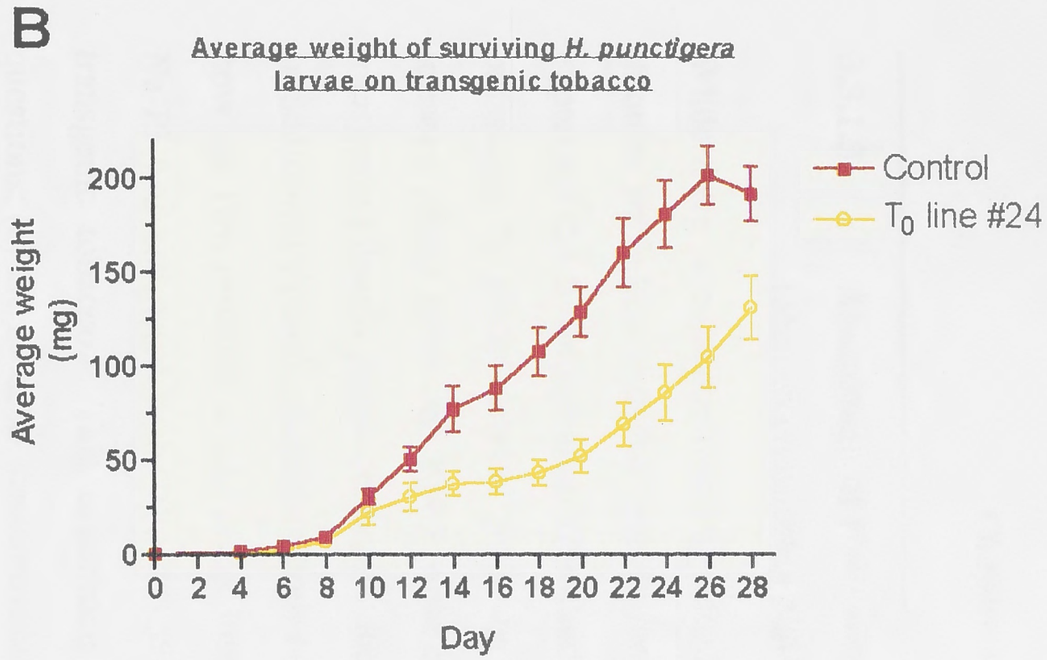
Fig. 3.4 Mortality and growth rate of *Helicoverpa punctigera* growing on transgenic tobacco or peas

A. Comparison of mortality of *H. punctigera* larvae growing on leaves of control or transgenic tobacco containing Na-PI, during a 24 day time period. Forty larvae were added to the leaves at day 0. B. Average weight of *H. punctigera* fed leaves from transgenic or control plants. Each data point is the average of a maximum of 40 or a minimum of 4 larvae (in the case of larvae on transgenic leaves) and vertical bars represent standard error of the mean (SEM). C. As for A, but *H. punctigera* were fed leaves from transgenic peas, or controls, respectively. D. Average weight of surviving *H. punctigera* grown on leaves of control or transgenic peas plants. Data points are the average from a maximum of 40 or a minimum of 28 larvae. Vertical bars are the SEM. *H. punctigera* mortality and development time increased for larvae feeding on transgenic tobacco or pea leaves, relative to larvae on control leaves.

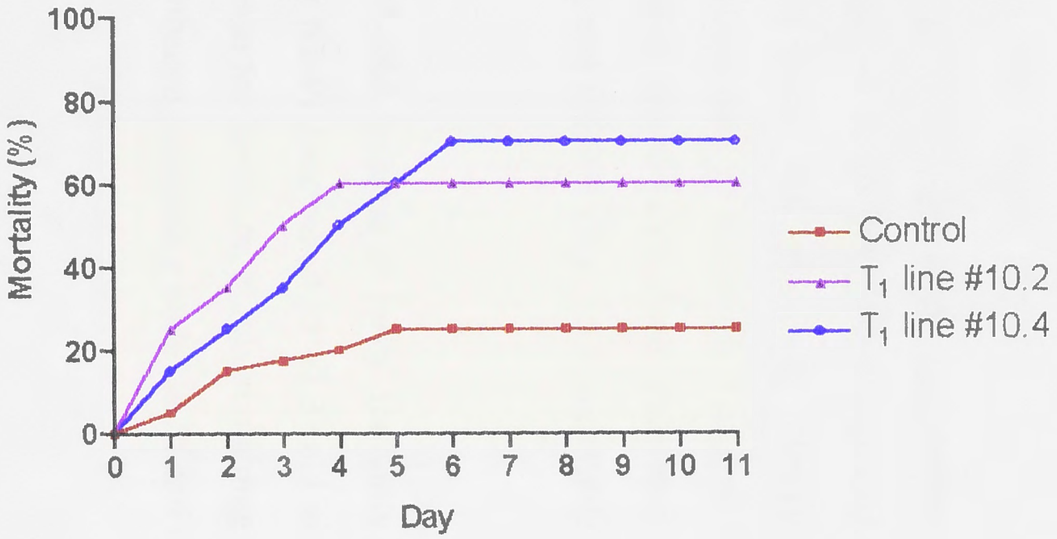
A


Mortality of H. punctigera larvae on transgenic tobacco
Mortality (%)
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Day
T0 line #24
Control

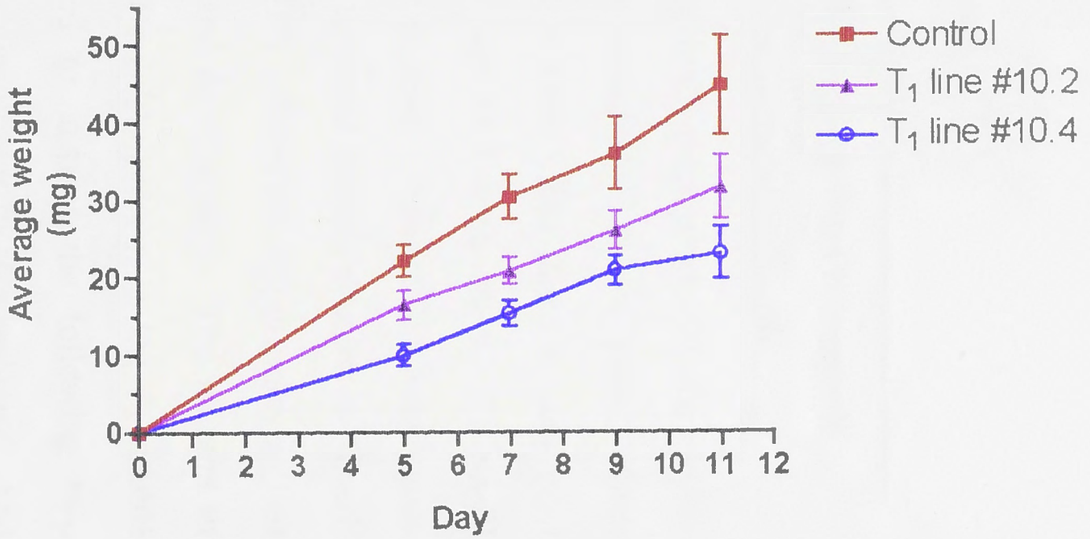
B


Average weight of surviving H. punctigera larvae on transgenic tobacco
Average weight (mg)
200
150
100
50
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Day
Control
T0 line #24

C


Mortality of H. punctigera larvae on transgenic pea
Mortality (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11
Day
Control
T1 line #10.2
T1 line #10.4

D


Average weight of surviving H. punctigera larvae on transgenic pea
Average weight (mg)
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Day
Control
T1 line #10.2
T1 line #10.4

3.3.1.3 Assessment of *Helicoverpa armigera* development on transgenic tobacco containing Na-PI, β-HTH alone or in combination

While there is evidence that PIs afford protection against insect pests, the toxicity of thionins to insects remains speculative. I produced transgenic tobacco plants which expressed the β-hordothionin from barley (see Chapter Two). For these *H. armigera* bioassays, T_2 plants derived from the homozygous line #1.3 were used. T_2 Na-PI tobacco plants from line #24.2 were also grown from seed. These T_2 homozygous transgenic tobacco plants contained about 0.38% of the total soluble protein as Na-PI. In addition, F_3 plants containing both Na-PI and β-HTH offered the opportunity to test how the two proteins would affect insect mortality and development. The amount of Na-PI was estimated to be about 0.5%, of the total soluble protein in the F_3 double transgenic tobacco. This experiment was designed to answer the following two questions: (a) are tobacco transformed with β-HTH more tolerant to *H. armigera*? And (b) does the presence of both Na-PI and β-HTH afford greater protection from *H. armigera* than either gene alone?

Larval mortality

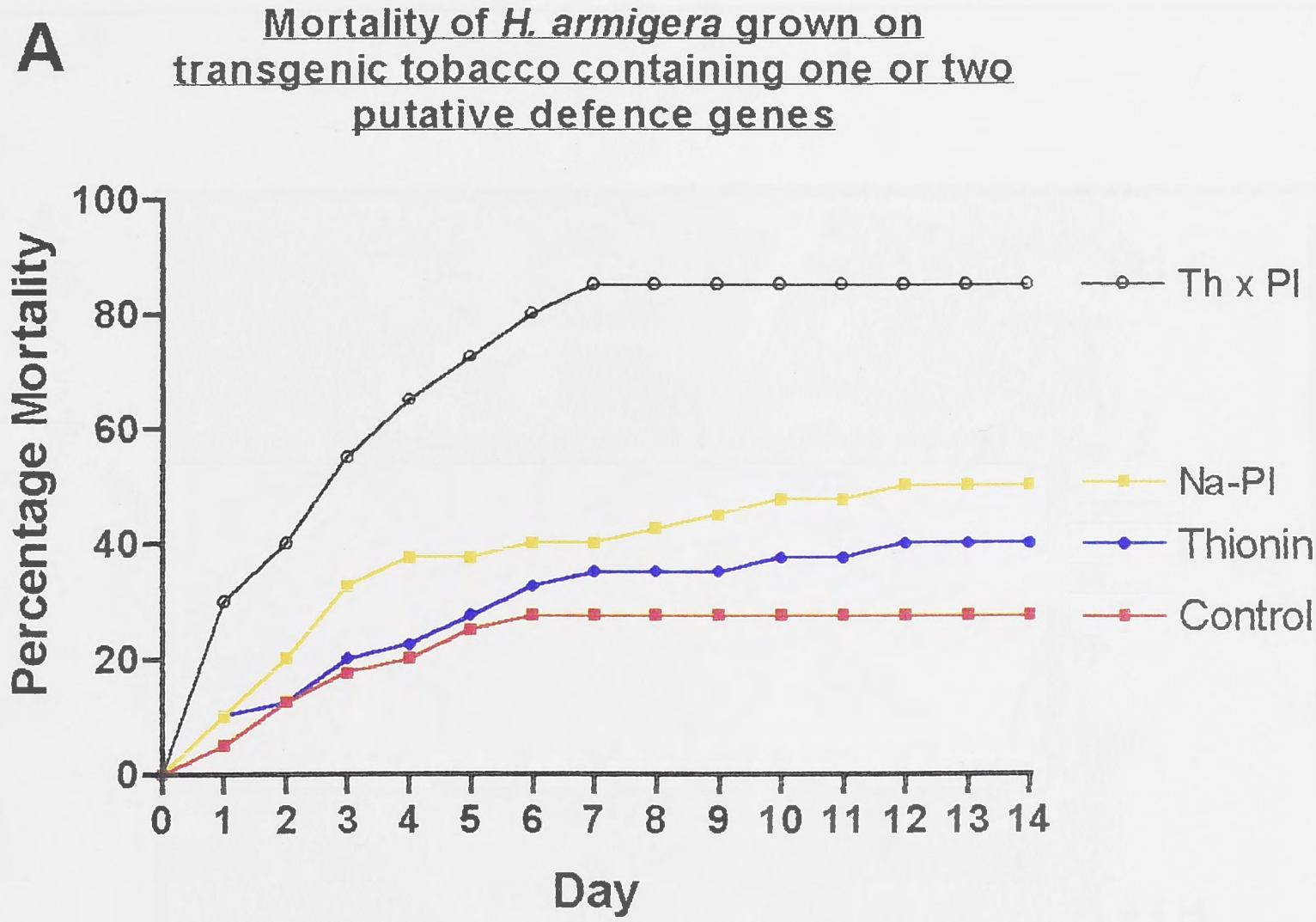
As in previous experiments, overall mortality was high in the first seven days of the experiment (Fig. 3-5A). About 28% of the *H. armigera* larval population ingesting control leaves died compared to 40% mortality for larvae fed transgenic tobacco containing β-HTH. At the end of larval development about 50% of the larvae died when fed leaves from Na-PI transgenic tobacco, whereas 85% of the larvae died when they were grown on tobacco transformed with both Na-PI and β-HTH (Fig 3-5A and Table 3-5).

In summary, this experiment showed that about a 1.4-fold increase in larval mortality could be attributed to β-HTH, a 1.8-fold increase for Na-PI (compared to ~2.3-fold in previous experiments; Table 3-1), and a 3.1-fold increase for larvae on plants containing both genes. In a second experiment using transgenic tobacco containing both Na-PI and

β-HTH genes, the high level of larval mortality was confirmed and was significantly different to control mortality ($P < 0.0001$). In this second experiment, there was a 3.7-fold increase in larval mortality on leaves from double transgenics (Table 3-5). On averaging the results of the two experiments, there was a 3-fold increase in *H. armigera* larval mortality in plants containing the two genes.

Growth rate and development of surviving larvae

H. armigera larvae on control leaves grew more quickly than larvae on transgenic leaves, and reached their peak weight at 14 days (Fig. 3-5B), which compared well with previous experiments (Fig. 3-2B). Although larvae growing on β-HTH transgenic leaves developed at the same rate as on control leaves, they weighed significantly ($P < 0.025$) less at the end of larval development and prior to pupation. Larvae on leaves from transgenic plants containing Na-PI accumulated the same final biomass as for larvae on control leaves, except that there was a two day developmental delay as peak weight was reached at 16 days. These results with T_2 plants confirmed earlier results using T_0 plant material. Interestingly, larvae on leaves from tobacco containing both Na-PI and β-HTH, showed a delay in development (of four days) and weighed significantly ($P < 0.001$) less at the end of larval development. Therefore, it appeared that Na-PI and β-HTH inhibited *H. armigera* larval development in an additive manner. Photographs of each *H. armigera* population were taken at day 8, to demonstrate both the decline in larval numbers and the relative size of larvae (Fig. 3-6A). A single larvae representing the size of each population at day 14 is also shown (Fig. 3-6B).



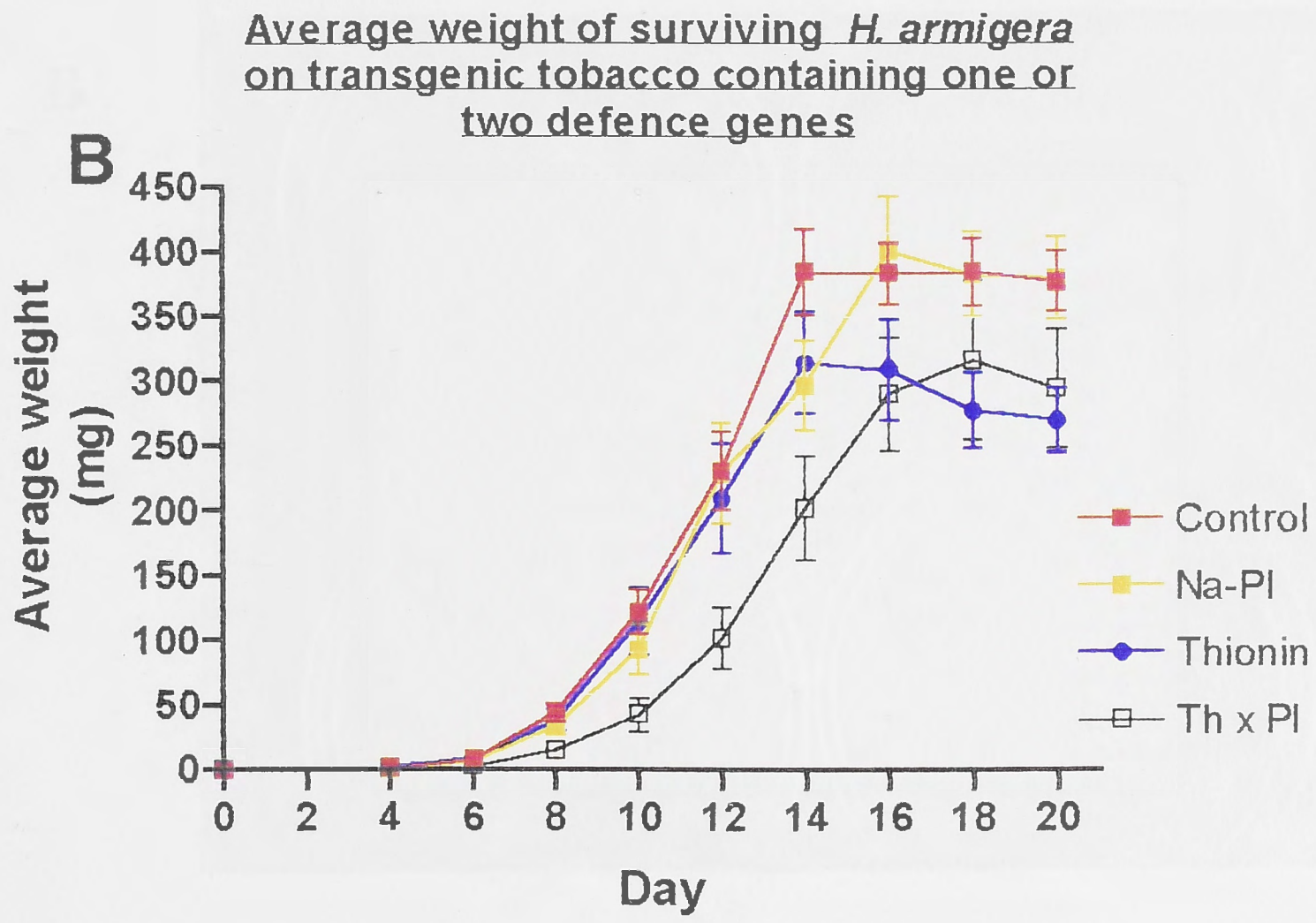
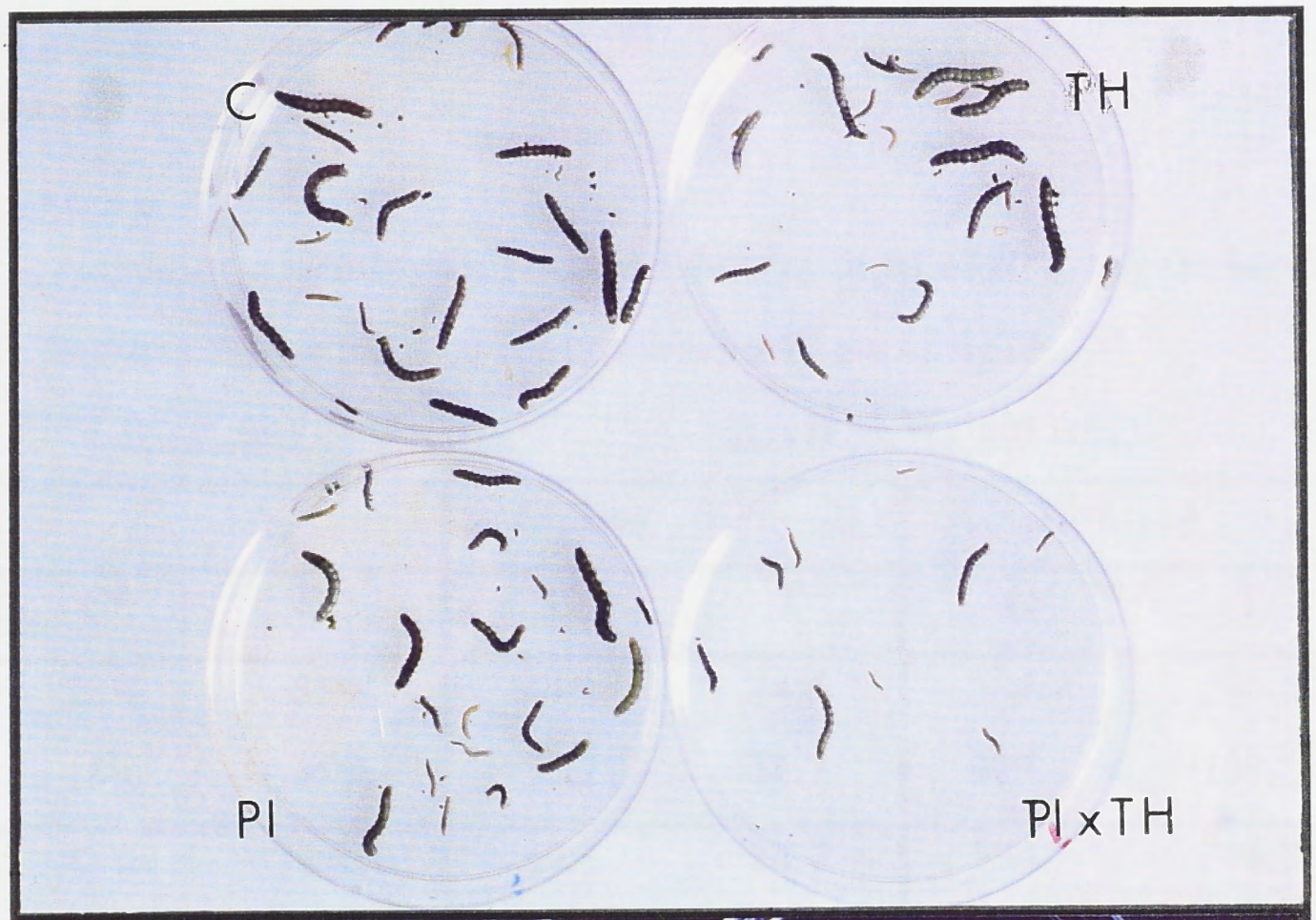


Fig 3-5. Mortality and growth rate of *H. armigera* larvae on control or transgenic tobacco containing one or two putative defence genes.

A. Cumulative percentage of larvae which died during a period of 14 days. Individual lines are as indicated by the key on the right hand side of the graph. Each treatment started with 40 neonate larvae.

B. Weight of surviving *H. armigera* larvae, each point represents the average weight of larvae in each population. Vertical bars are the standard error of the mean.

A



B

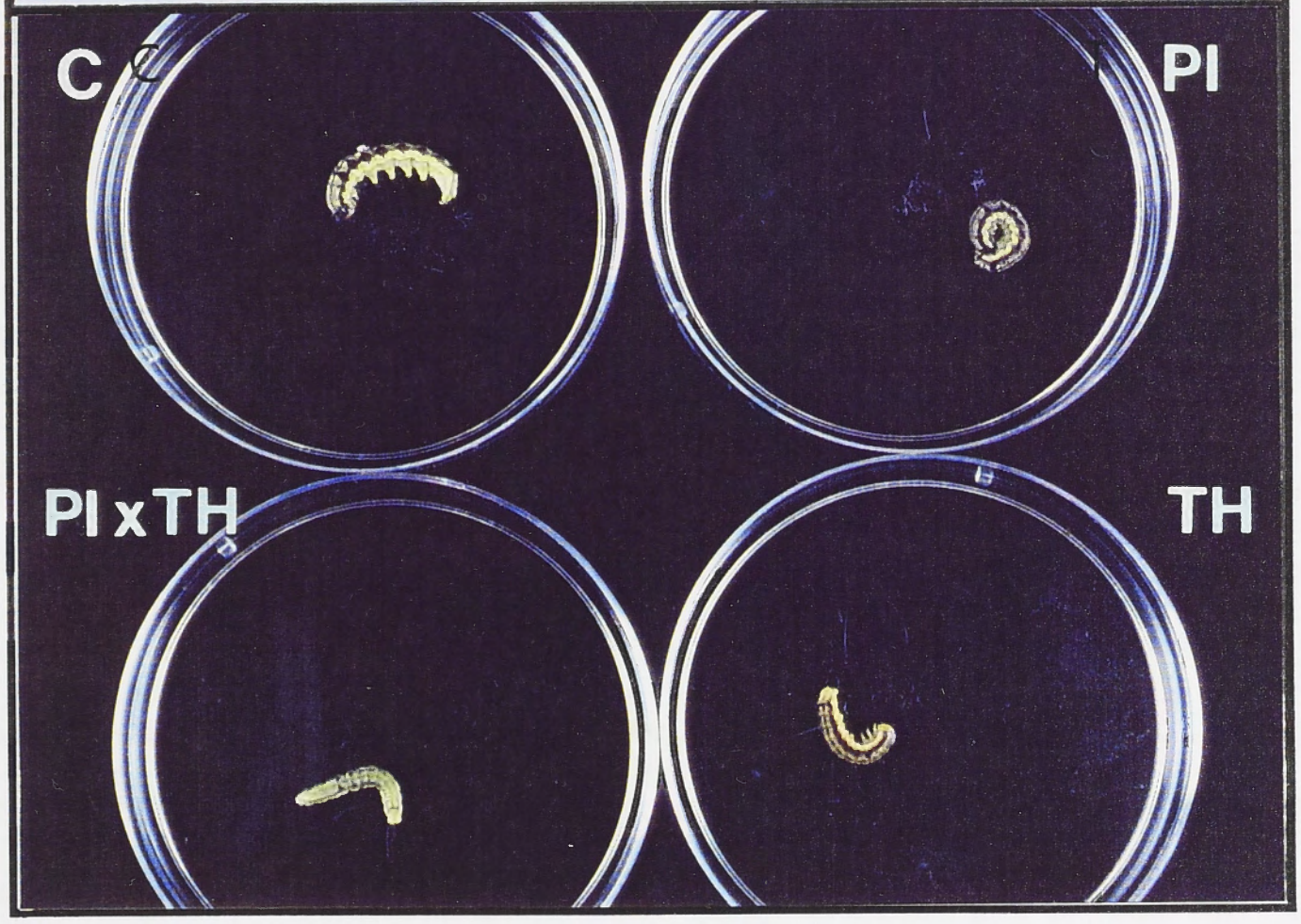


Fig. 3-6. Photographs of *H. armigera* larvae which had been grown on either control or transgenic tobacco leaves.

A. 8 day old populations of *H. armigera* larvae grown on leaves from either control or transgenic plants to show the relative number and size of larvae in each treatment. The label 'C' indicates larvae on control leaves. PI, TH and PI x TH represent Na-PI transgenic tobacco, β-HTH transgenic tobacco plants and double transgenics, respectively. B. Individual *H. armigera* larva chosen to represent the average size of each population at day 14. Labels for larva are as in A.

In the second experiment larval weights at day 8, were similar to those in experiment I (Table 3-5). As observed previously, the effect of Na-PI and β-HTH is prevalent early in development, as after 14 days, the margin between weights of control or transgenic fed larvae had closed.

Table 3-5. Increased mortality and delayed development of *H. armigera* larvae fed transgenic tobacco containing Na-PI and β-HTH

	Mortality		Average Weight (mg)			
			Day 8		Day 14	
Expt.	C*	T*	C	T	C	T
I	28%	85%	44.1	14.6	384.7	294.1
II	18%	65%	55.2	14.7	325.8	198.3
Ave.	**23%**	**72.5%**	**49.6**	**14.7**	**354.7**	**246.2**

*C and T denote control or double transgenic (Na-PI/β-HTH), respectively.

3.3.3 Feeding trial with redlegged earth mite

T_2 transgenic subclover lines #4.1 and #6.1 were chosen for feeding trials using RLEM because both of these lines were shown to be homozygous and accumulated 0.08% or 0.1% Na-PI, respectively. Subclover line #3.2 which had been transformed with the *na-pi* gene construct but contained undetectable amounts of Na-PI, was used as a control. Results from at least one experiment demonstrated Na-PI increases juvenile redlegged earth mite mortality, and appeared to affect behaviour as the number of mites feeding on transgenic plants was lower than the number feeding on control plants. For example, after 33 days of feeding, the number of mites feeding on transgenic line #4.1 was about half the number which fed on the control plants. The number of mites found on transgenic line #6.1, however, was higher relative to the number feeding on controls. Consequently, there was only half as much leaf tissue damaged for line #4.1 and a proportionately higher amount of damage to line #6.1. At this early stage, the results remain inconclusive and further experiments are underway.

3.4 Discussion

3.4.1 *Helicoverpa armigera* and *H. punctigera* bioassays

3.4.1.1 Effect of Na-PI on *Helicoverpa* species

A summary of the major conclusions derived from the experiments aimed at assessing tobacco or pea transformed with *na-pi,* against *H. armigera* or *H. punctigera* is given in Table 3-6.

Table 3-6. Summary of effects of transgenic plants containing Na-PI on *Helicoverpa* sp.

Species	Tobacco	Pea
H. armigera	• Average of 2.3-fold increase in mortality • 3 day developmental delay	• ~1.8-fold increase in mortality • 2 day developmental delay
H. punctigera	• ~3.3-fold increase in mortality • 7 day developmental delay	• ~2.6-fold increase in mortality • 3 day developmental delay

The presence of Na-PI in transgenic plants was associated with an increase in mortality of both *H. armigera* and *H. punctigera. H. punctigera* appeared to be more sensitive to Na-PI, as on average, the affect on mortality (relative to control fed larvae) was greater for this species, than for *H. armigera.*

For both *Helicoverpa* species, transgenic tobacco appeared more effective in causing larval death, than transgenic peas. These differences correlated with the relative levels of Na-PI which accumulated in the transgenic tobacco and pea plants, being nearly double in tobacco. Dose-dependant toxicity of PIs have been studied elsewhere in artificial diets using soybean trypsin inhibitor (SBTI). The mortality of *H. armigera* larvae was proportional to the amount of SBTI consumed and at the highest level tested (0.75mM), 100% of larvae died (Johnston *et al.,* 1993). However, in contrast to the study presented here (which used freshly harvested leaves), SBTI did not affect larval survival in the first 14 days and it was only after this time that mortality increased (Johnston *et al.,* 1993). On the other hand, mortality of poplar leaf beetle populations increased by

40% when fed poplar leaves expressing a cysteine proteinase inhibitor and over half of all deaths occurred in the first 15 days (Leplé *et al.,* 1995). The effect of Na-PI on larval mortality, when combined with slower development, lethargy and environmental stresses may be even more marked under field conditions.

Helicoverpa larvae which ingested Na-PI developed more slowly (Figs. 3-2 to 3-4) and the number of days by which development was delayed correlated with Na-PI expression. T_0 transgenic tobacco lines #13 and #24 contained 0.17% and 0.28% Na-PI, respectively. *H. armigera* which ingested leaves from line #13 required an additional two days to pupate while larvae on line #24 experienced a three day developmental delay. Similarly with peas, line #10.2 accumulated a lower amount of Na-PI than line #10.4. *H. punctigera* larvae growing on these lines were delayed in development by two days for line #10.2 and by four days for line #10.4. In addition, development of *Helicoverpa* species appeared to be more affected by transgenic tobacco than transgenic pea (see Table 3-6 for details), which is likely to be explained by the higher accumulation of Na-PI in tobacco, compared with peas. *H. punctigera* fed on transgenic tobacco or peas were seven or three days, respectively, behind in development, relative to control fed larvae. When compared to the shorter developmental delay (e.g. two or three days) experienced by *H. armigera* larvae, this also supports the earlier claim that *H. punctigera* were more sensitive to Na-PI.

The finding that Na-PI in transgenic plants slowed insect development extends the recent finding that the weight of *H. punctigera* larvae could be retarded by 54% after 12 days, when Na-PI was included in artificial diets (Heath *et al.,* 1997). While there are a number of reports that serine PI genes confer enhanced resistance to insect attack for transgenic tobacco (Hilder *et al.,* 1987; Johnson *et al.,* 1989; Boulter *et al.,* 1990; Thomas *et al.,* 1995a), it has also been shown that rice and cotton can be protected by the addition of PI genes. Rice plants have been transformed with potato PI I (Duan *et al.,* 1996) and cowpea trypsin inhibitor (CpTI) (Xu *et al.,* 1996). In both cases, the transgenic plants exhibited increased resistance to major insect pests of rice (Duan *et al.,* 1996; Xu *et al.,* 1996). Furthermore, cotton containing PIs from *Manduca sexta* were

protected against the sweet potato whitefly (Thomas *et al.*, 1995b). This work extends these observations and showed that PI genes can confer protection to peas from *Helicoverpa* species.

3.4.1.2 Combined effect of Na-PI and β-HTH on *Helicoverpa armigera*

While PIs in transgenic plants have been shown to improve resistance to pests, the use of this single strategy must be considered carefully. There is evidence that insects adapt to PIs by synthesising proteinases which are insensitive to the introduced PI (Boulter and Jongsma, 1995; Broadway, 1996). For example, Jongsma *et al.*, (1995) showed that *Spodoptera exigua* larvae fed transgenic tobacco containing PPI II could overcome the effects of the inhibitor by synthesising a new trypsin protease activity which was insensitive to PPI II. A further concern in the use of PIs alone in transgenic plants is that larvae are thought to exhibit decreased sensitivity to PIs later in the lifecycle (Orr *et al.*, 1994) which may potentiate the development of resistance to PIs.

One way to slow the development of insect resistance is to use PIs in alliance with other insecticidal molecules with different modes of action. Transgenic tobacco showed improved resistance when Na-PI and β-HTH were used in combination and larval mortality increased by 3.1-fold, relative to control plants. The effect of the two genes appeared to be additive because *H. armigera* larvae were both slowed in development (a characteristic of Na-PI ingestion), and were smaller prior to pupation, a characteristic of larvae on tobacco containing only β-HTH.

Although pyramiding a proteinase inhibitor and thionin had not been tested previously, other workers have investigated combined interactions of alternative defence molecules. For example, the efficacy of *Bacillus thuringiensis* toxins was enhanced by serine proteinase inhibitors in an *in vitro* study (MacIntosh *et al.*, 1990), and this provides some evidence that this strategy may be effective in further protecting plants from insect attack. In another case, the effects of transgenic tobacco plants containing both CpTI and pea lectin, were additive in protecting tobacco from *H. virescens* (Boulter *et al.*, 1990).

In conclusion, because the combined effects of Na-PI and β-HTH in transgenic tobacco enhanced their resistance to *H. armigera* these defence molecules should be considered among the suite of genes available for transfer into crops, either alone or preferably in combination with other defence molecules.

3.4.2 RLEM bioassays

Whole plant experiments were used to test if transgenic subclover expressing Na-PI were more tolerant to RLEM. The experimental conditions were aimed at closely representing field conditions (James Ridsdill-Smith, pers. comm.). For example, in the field, when RLEM are not feeding on plant cotyledons, they dwell predominantly in the soil. In this way, the behaviour of the mites can be observed. In at least one experiment, there appeared to be an interaction between Na-PI and RLEMs. At this early stage of analysis, it is not clear how this interaction should be interpreted in relation to the biology of mite development. For example, it is possible that Na-PI is detrimental to RLEM growth, but only during the first developmental stages (Garrick McDonald; pers. comm.). A delay in RLEM development may influence other growth parameters such as number of mites feeding on plants, reproduction, fecundity and leaf damage.

In conclusion, the production of transgenic subclover containing Na-PI was an important contribution to the research programme aimed at implementing integrated management of RLEM. The availability of transgenic material established collaborations with CSIRO, Division of Entomology, Perth and Agriculture Victoria, by providing co-workers with an opportunity to assess these lines for improved resistance. Although at this time, inference can not be drawn from the results, further work is in progress in Perth and Victoria. The production of genetically engineered subclover, in combination with research efforts by plant breeders, could eventually allow the development of new lines with host resistance.

3.5 Chapter summary

Transgenic tobacco and pea expressing Na-PI proved insecticidal for both *Helicoverpa armigera* and *H. punctigera* by increasing larval mortality and slowing development by

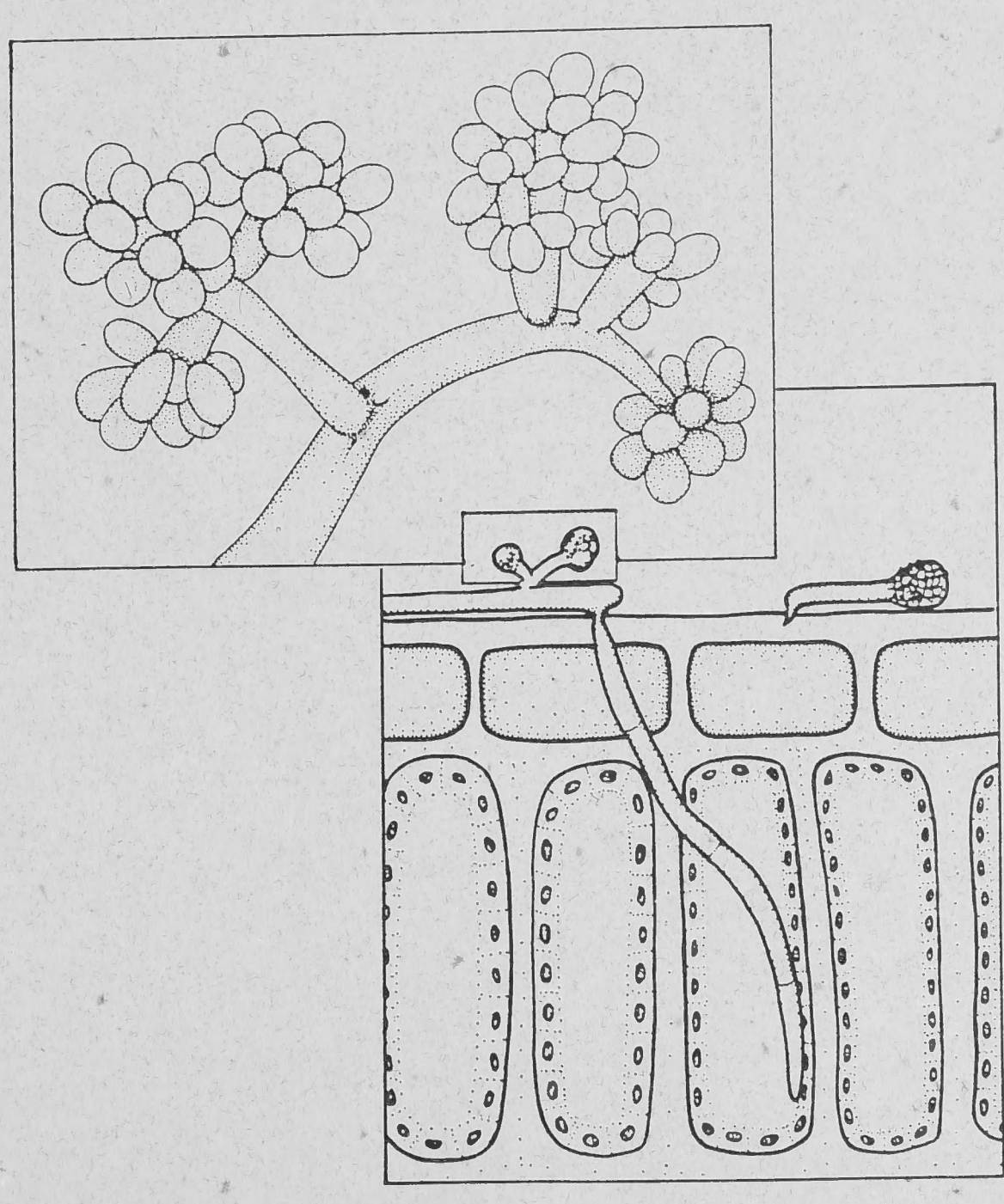
up to seven days. For both plant species the level of protection correlated to the relative amounts of Na-PI expressed in leaves. Furthermore, transgenic plants were better protected against *Helicoverpa punctigera,* as this pest appeared more sensitive to Na-PI, than *H. armigera.*

The mortality of *H. armigera* which ingested leaves from transgenic tobacco containing Na-PI and β-hordothionin, in combination was higher, than for *H. armigera* fed leaves from plants containing either gene alone. The effect of two genes appeared additive, for example, mortality of larvae fed transgenic plants with Na-PI or β-HTH alone, increased ~1.8- or 1.5-fold, respectively, whereas mortality rose 3.1-fold for *H. armigera* fed tobacco transformed with both genes. In addition, larvae grown on the leaves of double transgenic plants weighed significantly less and developed more slowly, relative to larvae on control leaves.

There is anecdotal evidence that transgenic subclover increases juvenile redlegged earth mite mortality, however the nature of the interaction between Na-PI and the mites remains unclear. There are more thorough tests underway which are aimed at determining the significance of this finding.

Chapter Four

Pathogen Bioassays



Chapter Four

Assessment of transgenic plant performance using fungal and bacterial bioassays

4.1 Introduction

The ultimate goal of plant pathology is to understand and effectively manage disease. Improving yield and productivity of crop plants by increasing disease resistance is a major goal of breeding programmes. Current methods of disease management in Australia include both regulatory procedures and farm management practices. Mandatory quarantine schemes aim to eradicate disease, particularly from imported seeds and cuttings and farm practices, like sanitation, crop rotation, improved drainage and irrigation are important mechanisms to keep pathogens in check. Chemicals applied as sprays, dusts, seed and soil treatments are other means to control diseases, they are cost-effective and have led to few cases where pathogen resistance has developed. However, their effectiveness depends on how uniformly the chemical is sprayed, and new foliage is not protected. In addition, many chemical pesticides have been withdrawn because of human and environmental toxicity. The development of fungicides is still challenging, as over 20 types of toxicological and environmental safety tests are required before release. Moreover, it can cost $86M to market a new chemical, which gives patent protection for 16 years, allowing only 12-14 years to recoup development costs before a profit is made (Agrios, 1988).

Together, these factors have helped initiate a drive for a decreased reliance on chemical fungicides, better utilisation of integrated pest/pathogen management strategies and an increase in research to develop new technologies, that would provide plants with in-built protection from disease. One biotechnological approach to increase disease resistance, is

to genetically engineer plants with genes which encode putative defence factors. Several sources of genes for pathogen resistance have been identified and new ones are being discovered. The most common are genes found in plants which encode for proteins with antimicrobial activity. Among them are proteins that are induced upon pathogen attack like chitinases, β-1,3-glucanases and proteinase inhibitors. Others include small antimicrobial peptides which may be present constitutively like the thionins, lipid-transfer proteins and plant defensins (see Chapter One for review).

Reports which documented the antifungal activity of thionins and proteinase inhibitors (PIs) provide evidence that these proteins could be suitable candidate genes for pathogen protection. Firstly, thionins are induced in leaves after fungal infection (Bohlmann *et al.,* 1988), and secondly, thionins inhibit the growth *in vitro,* of both plant pathogenic bacteria and fungi (Cammue *et al.,* 1992; Florack *et al.,* 1993). Likewise for PIs, a role in plant defence is implied because they are induced upon wounding (Green and Ryan, 1972; Pearce *et al.,* 1993), and attack by pathogens (Peng and Black, 1976; Gatehouse *et al.,* 1979; Geoffroy *et al.,* 1990).

To substantiate the claims that thionins and proteinase inhibitors are potential important defence molecules, other lines of evidence are required. A direct test for anti-pathogen activity is to introduce the genes for these proteins into plants and demonstrate that transgenic plants containing the new trait have enhanced resistance to pathogens. In one study, tobacco plants transformed with an α-hordothionin gene were more resistant to infection by *Pseudomonas syringae* pv. *tabaci* or *P. syringae* pv. *syringae* as evidenced by a reduction in lesion symptoms (Carmona *et al.,* 1993b). However, other workers have claimed that transgenic tobacco expressing α-hordothionin are not protected against *P. syringae* pv. *tabaci* (Florack *et al.,* 1994). On the basis of these contradictory results, it was clear that further work was needed. Moreover, there are no reports of transgenic plants containing PI genes which have been tested for improved resistance to fungal or bacterial pathogens. It appeared, therefore that there were substantial gaps in

the understanding of the potential of thionins or PIs genes to confer resistance to pathogens.

To this end, I transferred the cDNA encoding genes for a proteinase inhibitor from *Nicotiana alata* (Na-PI) and a β-hordothionin (β-HTH) from barley endosperm into tobacco. This chapter describes experiments aimed at assessing transgenic tobacco plants for improved tolerance to fungal and bacterial pathogens. Any strategy to test if Na-PI and β-HTH provide enhanced disease resistance in plants, must include suitable pathogen bioassays. Criteria in selecting possible fungi and bacteria for this study stipulated that they; a) be highly infectious, so that differences in resistance could be scored easily; b) be pathogens of considerable importance to industry and; c) are likely to be sensitive to thionins and/or PIs, based on previous evidence.

Preliminary experiments with a range of fungi, using leaf disk bioassays demonstrated that some were more suitable as infectious agents than others. *Chalara elegans* and *Phoma pulmoron* although pathogens of tobacco, were unreliable in their infectivity of detached leaf disks (data not shown). Further experiments tested the efficacy of transgenic T_0 line #24, containing Na-PI against *Phytophthora nicotianae* var *nicotianae* (strain 4975) (stem black shank disease). Na-PI had only a slight detrimental effect on the growth of this fungi, but this was not significantly different from controls (data not shown). One fungus which fulfilled the selection criteria was *Botrytis cinerea* - an air-borne pathogenic fungus responsible for considerable losses in many crops throughout the world (Jarvis, 1980). *B. cinerea* or 'grey mould', is a necrotrophic fungus (kills the host cells to obtain nutrients for growth). There are more than 200 host plants for *B. cinerea,* such as soft fruits, vegetables, ornamental flowers and tree seedlings (Jarvis, 1980). Commonly infected fruits are strawberries, raspberries, tomatoes, sweet peppers, apples, pears and grapes (Fig. 4-1) (cited in Bossi *et al.,* 1994). *B. cinerea* also readily infects tobacco leaves, if a small wound is made on the surface. Robin and Guest, (1994) developed a bioassay whereby the growth of this fungus can be monitored by measuring the size of the necrotic lesion which forms around the site of infection. What's more, and important for this overall strategy, is that as part of the invasion process, *B. cinerea*

secretes trypsin- and chymotrypsin-like proteases (Brown and Adikaram, 1983). Consequently, it is possible that growth of this fungus could be inhibited by the trypsin and chymotrypsin inhibitors derived from Na-PI. The *B. cinerea* bioassay would also prove a useful tool in determining the effect of β-HTH on fungal growth in transgenic tobacco. Again, the toxicity of thionins *in vitro* was the driving force for these experiments (e.g. Bohlmann *et al.,* 1988). In addition, transgenic tobacco transformed with both Na-PI and β-HTH were tested for improved resistance to *B. cinerea* as a means to provide valuable information about the interaction between the two defence genes. This chapter describes the results of bioassays which assessed the sensitivity or tolerance of transgenic tobacco to *B. cinerea.*

The second bioassay used *Pseudomonas solanacearum,* which is an important bacterial pathogen that has a major impact on the production of economic crops such as tobacco, potato, tomato and groundnut. There have been many reports of increasing incidence of this disease particularly in Asia, Africa and the South Pacific in the early 1990s highlighting the increasing seriousness of the pathogen throughout the world (Mehan and Liao, 1994). In Australia it is most prevalent in Queensland, affecting potatoes, ginger, tomato, capsicum, eggplant and tobacco. The destructiveness of *P. solanacearum* is compounded by its wide host range (30 families of monocotyledons and dicotyledons) and its persistence in different types of soils (Graham *et al.,* 1979). It is a motile, non-sporalative, gram-negative bacterium. Infection usually occurs through the roots, but the pathogen can also enter through wounds on the stem, through the stomata or through the stolons on potato tubers (Dowson, 1957). Rapid progression of the disease results in the destruction of xylem tissues and blocks the production of tyloses thus compromising the vascular system and causing the plant to wilt (Buddenhagen and Kelman 1964; Wallis and Truter, 1978); hence its common name 'bacterial wilt'. In addition, *P. solanacearum* produces extracellular polysaccharides which interfere with water movement, especially in the leaves (Hussain and Kelman, 1958; Wallis and Truter, 1978). Vascular tissue is also destroyed by extracellular enzymes, like pectinases and hydrolases which the bacterium secretes during the infection process (Kang *et al.,* 1994).

On tobacco, the disease is called "Granville Wilt". The leaves wilt and turn yellow, roots and stem bases rot and in severe cases the plants are significantly stunted (Fig. 4-1B) and bacteria are observed oozing from the stem. Tobacco seedlings are extremely susceptible to *P. solanacearum,* and can be infected simply by injecting a liquid bacterial culture into the soil surrounding the roots. This procedure allowed the development of a reliable and reproducible bioassay for assessment of transgenic plants for improved resistance (Peter Hughes, pers. comm.). For *P. solanacearum*, the best chance of attaining resistant tobacco, were those plants which contained β-HTH, either alone, or in combination with Na-PI.

This chapter describes experiments testing the effectiveness of transgenic tobacco containing Na-PI and β-HTH, alone or in combination against the fungal pathogen *Botrytis cinerea* and the bacterial pathogen *Pseudomonas solanacearum* because there is evidence that Na-PI does not effectively inhibit this bacterium *in vitro* (Dunse, 1992).

A

B

Fig. 4-1 Disease symptoms of *Botrytis cinerea* and *Pseudomonas solanacearum*.
A. *B. cinerea* infection on grapes, causing an effect known as 'raisining' (from Sall *et al.*, 1982). B. *P. solanacearum* infection on tobacco, infected plant in the foreground and healthy plant in the background (from Vock, 1978).

4.2 Materials and Methods

4.2.1 Leaf disk *Botrytis cinerea* bioassay

4.2.1.1 Selection of tobacco lines for bioassay

Transgenic plants were T_1 tobacco lines #13.2 and #24.2, which had been transformed with Na-PI and tobacco line #1.3 transformed with β-HTH, and the F_2 line #5.17, containing both Na-PI and β-HTH. Hereafter, these lines are referred to as PI:#13.2, PI:#24.2, TH:#1.3 and PI x TH, respectively. Plants transformed with only the *bar* gene acted as a control. All plants were clonally propagated in tissue culture in media containing 1 mg/L benzylamino purine and 0.5 mg/L indoleacetic acid. Tobacco plants were rooted on hormone free media for three weeks before transferring to compost and maintained under natural light in a glasshouse at a 24°C day /12°C night temperature regime. Three plants for each treatment were placed randomly on one of three benches and were grown to maturity in the glasshouse. (Fig. 4-2). Leaves from six week old, 30 cm high, tobacco plants were used for bioassays.

4.2.1.2 Growth and maintenance of *Botrytis cinerea*

B. cinerea (strain 93.40) isolated from strawberries in Brunswick, Victoria, Australia was obtained from Dr. D. Guest, (School of Botany, University of Melbourne, Victoria) and maintained in the dark on V8 agar (20% V8 juice, 2% agar, and 0.2% $CaCO_3$) plates at 24°C. For bioassays, 10 identical plates of *B. cinerea* were established in the following way: a sterile scalpel was used to cut small blocks of agar with hyphae attached, from the margin of four day old cultures and placed on fresh 20% V8 agar. After allowing the fungus to grow for four days, a sterilised 4 mm diameter cork borer was used cut plugs of agar (and hyphae) from the growing edge of fresh cultures for inoculation of leaves.

4.2.1.3 Leaf-disk inoculation

I adapted the procedures which had been developed by Nemestothy and Guest, 1990 and Robin and Guest 1994 (see schematic diagram; Fig. 4-2 or Fig. 4-4A). Three young,

fully expanded leaves from each plant were labelled 'A-C' with leaf 'A' being the smallest and leaves 'B' and 'C' being consecutively larger down the stem of the plant. Leaves were excised and rinsed in running water for 10 min. One 4 x 4 cm leaf piece was cut from the left hand side of each leaf and a 3 mm hole made in its centre, taking care to avoid veins. Leaf pieces were placed randomly abaxial side up, in plastic boxes (15 x 10 x 5 cm) on sterile Whatman No. 1 filter paper moistened with 10 ml of water. The 4 mm mycelial plugs of *B. cinerea* (section 4.2.1.2) were inverted and placed over the central hole in the leaf. Control plants were similarly treated with sterile agar plugs. The boxes were sealed and incubated at 27°C in a growth cabinet with 16 h light, 8 h dark, except in the third experiment where conditions were 24°C, in the dark. The diameter of the necrotic lesion was measured using an electronic digital calliper at three time points at least 24 h apart until it appeared the lesions had stopped growing. If the lesion was not circular, the diameter was estimated by measuring the minimum and maximum widths of the lesion. The experiment was repeated three times.

4.2.1.4 Analysis of data

The lesion area was calculated from the estimated diameter. The student's t-distribution was used for preliminary two-way comparisons of the differences in lesion area between different treatments (Control, PI: #13.2, PI: #24.2, TH: #1.3 or PI x TH). Differences in lesion area which developed on the leaves of different treatments, for experiment I, were also analysed by analysis of variance (ANOVA). Additional sources of variation in the ANOVA were a) variation between plants (e.g. blocks 1-3) and b) variation between leaves (e.g. A-C), and box effects (e.g. 1-9). A simple ANOVA, testing the main effects was calculated using the GENSTAT® statistical package (Lawes Agricultural Trust, Rothamsted Experimental Station) by Roger Morton, Division of Water Resources, CSIRO.

4.2.2 Whole plant *Botrytis cinerea* bioassay

4.2.2.1 Whole plant inoculation

The same five plant lines used in the leaf-disk bioassay, were generated clonally for the whole plant bioassays. Briefly, there was an empty vector control, T_1 lines PI: #13.2; PI: #24.2; TH: #1.3 and F_2 line #5.17 with both Na-PI and β-HTH (PI x TH). In a second whole plant experiment, T_2 progeny derived from lines PI #24.2; TH #1.3 and F_3 line #5.17 were used (Table 4-1). As in section 4.2.1.1, three tobacco plants were grown for each treatment and were randomly placed on three benches (Fig. 4-2).

B. cinerea was plated on fresh V8 agar and four day old cultures were used in whole plant bioassays as described in section 4.2.1.2. Three young, fully expanded leaves from each plant were labelled 'A-C' using a marker pen. Four, 3 mm holes were made in each leaf, taking care to avoid veins. The area surrounding the hole was moistened with water and 4 mm mycelial plugs of *B. cinerea* and agar were inverted and placed over each hole. Control plants were sham-inoculated with sterile agar plugs. A paper tissue was immersed in water, and wrapped around the base of the leaf, as close to the stem as possible. A plastic bag was placed over the entire leaf and secured at the base, over the paper tissue, using plastic coated wire (see Fig. 4-5A and B). After three days, the plastic bags were removed and the diameters of the necrotic lesions were measured as described in section 4.2.1.3. The experiment with T_1 plants (and F_2 PI x TH) was repeated three times. The second experiment with T_2 plants and F_3 PI x TH was performed only once as the three experiments with T_1 plants were consistent. Statistical analysis for whole plant bioassays was as described in section 4.2.1.4 for the bioassays using leaf disks.

4.2.2.2 Microscopy

On the third day after inoculation, leaf pieces infected with *B. cinerea* were examined by compound microscopy. Leaf pieces were cleared to remove interfering leaf pigments by placing in lactic acid: absolute ethanol (3:1 v/v) for 48 h at room temperature. Sections

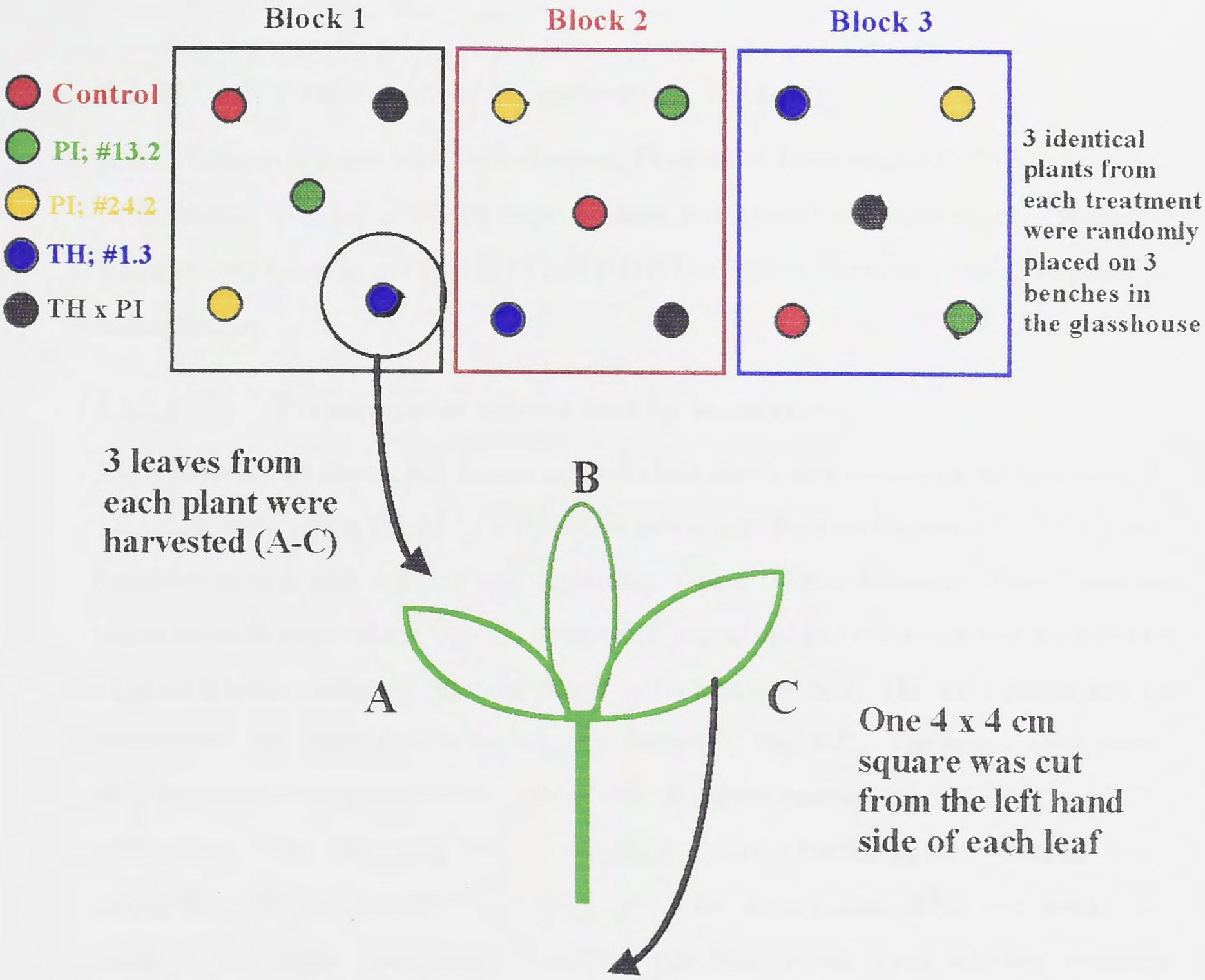
of leaf were dissected and mounted in lactophenol-cotton blue to observe the hyphal branches within the leaf cells.

In addition, photographs were taken using the stereo light microscope (Wild Leitz M8) by Stuart Craig, Division of Plant Industry, CSIRO. Scanning electron microscopy (Craig and Beaton, 1996) was used to examine morphological differences between the *B. cinerea* mycelium growing on control leaves and on transgenic leaves containing both Na-PI and β-HTH.

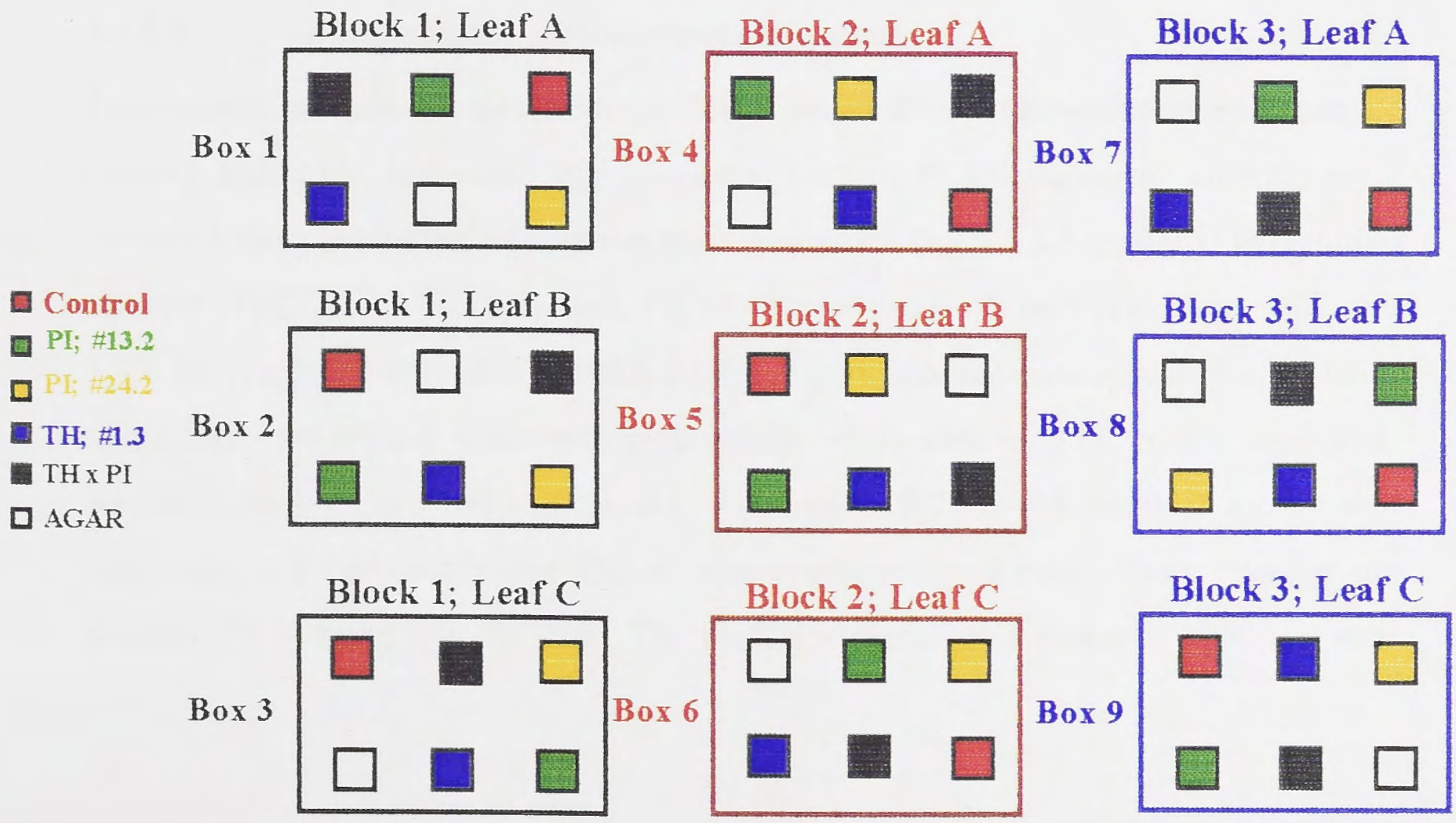
Table 4-1. Summary of plant material used in *Botrytis cinerea* bioassays

Bioassay	Plant Material	Generation	No. of Experiments
Leaf disk	Control (*bar* alone)	-	3
	PI: #13.2	T_1	
	PI: #24.2	T_1	
	β-HTH: #1.3	T_1	
	Na-PI x β-HTH: #5.17	F_2	
Whole plant	Control (*bar* alone)	-	3
	PI: #13.2	T_1	
	PI: #24.2	T_1	
	β-HTH: #1.3	T_1	
	Na-PI x β-HTH: #5.17	F_2	
Whole plant	Control (*bar* alone)	-	1
	PI: #24.2	T_2	
	β-HTH: #1.3	T_2	
	Na-PI x β-HTH: #5.17	F_3	

Fig. 4-2 Diagrammatic representation of the *Botrytis cinerea* bioassay
The three blocks are individual benches in a glasshouse, and each of the coloured circles represents a single tobacco plant, randomly assigned to one of five positions on the bench. Three leaves from each tobacco plant were harvested and labelled A, B or C. One 4 x 4 cm section was cut from each leaf and placed randomly in sealed, plastic boxes. A small wound on each leaf piece was inoculated with an agar plug containing *B. cinerea* hyphae. Each box contained a leaf piece from each of the five different plant lines, as well as a control leaf, sham inoculated with sterile agar. The experiment was designed to assess the variation of *B. cinerea* lesions, between plants from blocks 1-3, as well as between leaves (A-C), from the same plant.



Leaf squares from Block 1; leaf A were placed in a humidity box and sealed. All squares from Block 1; leaf B were placed in a separate box; etc.



4.2.3 *Pseudomonas solanacearum* bioassay

In a collaborative study with Peter Hughes, Division of Plant Industry, CSIRO (a fellow Ph.D student working on wheat α-purothionin and *Arabidopsis* lipid transfer proteins), we examined the potential for Na-PI and β-HTH to protect transgenic tobacco against *P. solanacearum.*

4.2.3.1 Preparation of tobacco lines for inoculation

Approximately 20 seeds each from a control plant which only contained the *bar* gene, T_2 TH: #1.3, and F_3 PI x TH #5.17 were sown into plastic boxes with lids (13 x 11 x 9 cm) half-filled with a well draining soil containing a slow release fertiliser. There were 40 boxes for each of the three tobacco lines which were arranged randomly into four blocks - i.e. each block contained 10 control boxes, 10 boxes with T_2 TH: #1.3 plants and 10 with F_3 PI x TH plants (see schematic representation: Fig. 4-3). The boxes were placed on a bench in an enclosed growth cabinet with day/night temperatures of 28°C and 25°C, respectively. The light cycle was 16 h light, 8 h dark. Seedlings were watered every second day and lids remained closed throughout the experiment. After two weeks, the plants in the boxes were thinned so that only four, equal sized tobacco seedlings remained. Seedlings were approximately 4-5 weeks old (6 cm high with about four leaves) when inoculated with *P. solanacearum.*

4.2.3.2 *Pseudomonas solanacearum* inoculum

Pseudomonas solanacearum (strain 6397) was obtained from Queensland Department of Primary Industries, Mareeba. For bioassays, virulent *P. solanacearum* colonies were identified using colour indicator plates containing 0.2% (w/v) 2,3,5-triphenyl tetrazolium chloride (TZC), 0.5% (v/v) glycerol, 1% (w/v) peptone, 0.1% (w/v) casamino acids and 1.8% (w/v) agar (Jenkins and Kelman, 1976). Pathogenic colonies appeared irregular in shape and were creamy white with pink-orange red centres on TZC media while non-virulent colonies were red and circular. A single 48-72 h old, virulent colony was suspended in a flask containing 250 ml semi-synthetic liquid media (from Hussain and Kelman, 1958) using a sterile loop. The flask was placed on a shaker at 28°C and after

~72 h, the inoculum was diluted (1:4) with Milli-Q Plus (Millipore) water for use in the bioassays.

For long-term storage of *P. solanacearum,* a single pathogenic colony from a 48-72 h old culture was suspended in 8 ml of water and stored at 20°C. Cultures stored in this way remain stable for up to two years (Jenkins and Kelman, 1976).

4.2.3.3 Inoculation and assessment of resistance

2 ml of diluted inoculum was injected into the soil within 1 cm surrounding each plant using a 0.8 x 38 mm hypodermic needle (Terumo) attached to a 2.5 ml syringe barrel (Terumo). In previous experiments, boxes containing control plants were inoculated in the same way, but with sterile liquid media. Each box was sealed with masking tape to prevent the soil drying out and the boxes were not watered during the bacterial growth phase.

After seven days, the number of plants in each box showing any symptoms was recorded as a percentage of the total number of plants in each treatment (160). After 14 days, each plant was classified according to a Disease Index where a score of 0 indicated plant death and a score of 4 were plants which only had one wilted leaf.

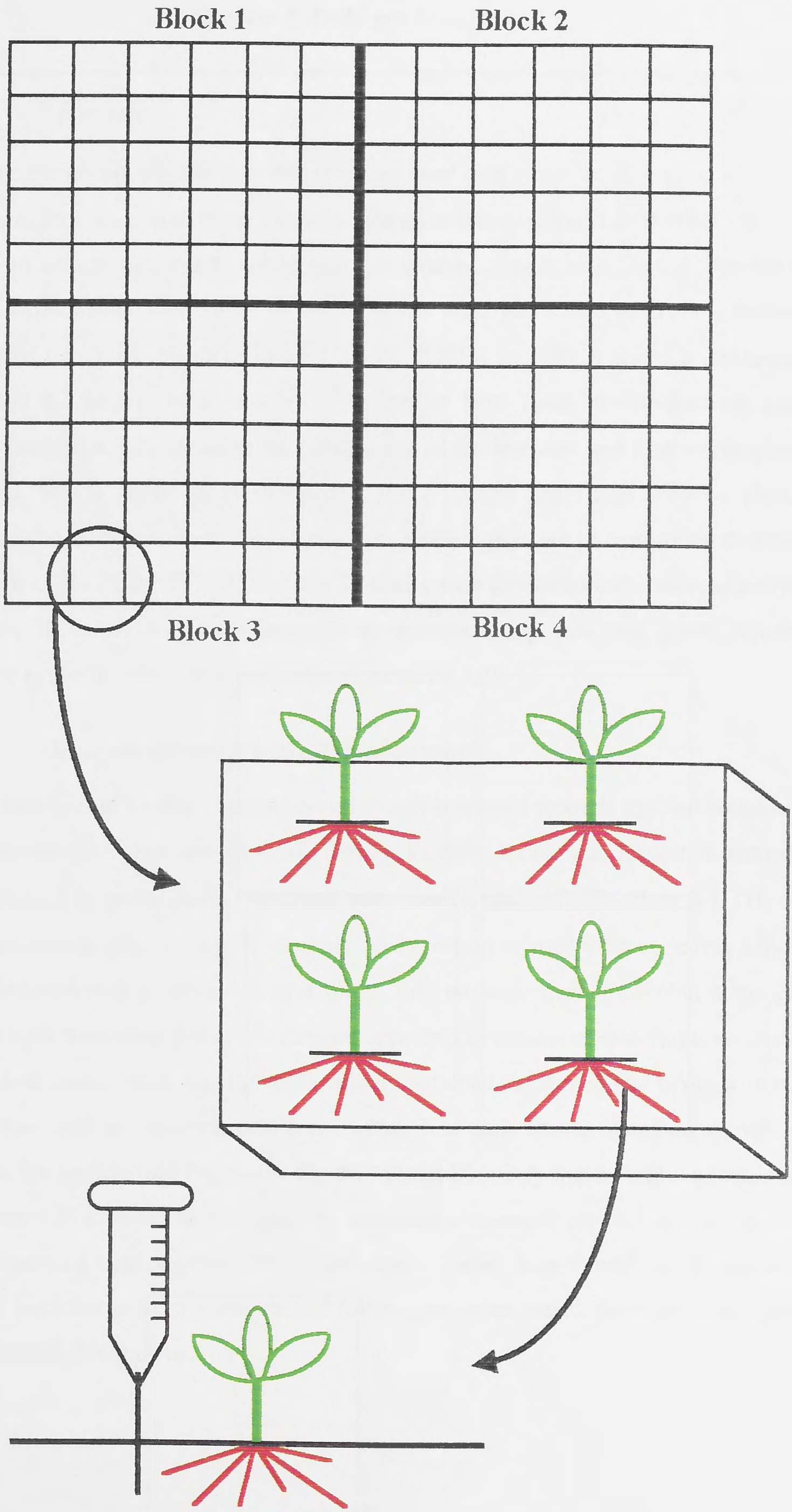
The entire experiment was repeated, although only the results from the second experiment are presented, as the rate of infection was higher.

4.2.2.4 Statistical analysis

The average percentage of plants showing symptoms in each box was calculated and standard errors were estimated using the box as the unit (rather than each plant). The difference between the percentage of tobacco with symptoms in each block was compared using the student's *t*-Test.

Fig. 4-3 Whole plant bioassay to the effect of Na-PI and β-HTH transgenic tobacco plants on resistance to *Pseudomonas solanacearum*

A bench in a growth chamber was divided into four blocks. Each block contained 30 boxes of plants which were randomly positioned - 10 control boxes, 10 boxes of β-HTH transgenic plants (T_2 line #1.3) and 10 boxes PI x TH plants (F_3 line #5.17). Each box contained four tobacco seedlings. The soil surrounding the roots of each seedling was injected with a dilute liquid culture of *P. solanacearum*.


Block 1
Block 2
Block 3
Block 4

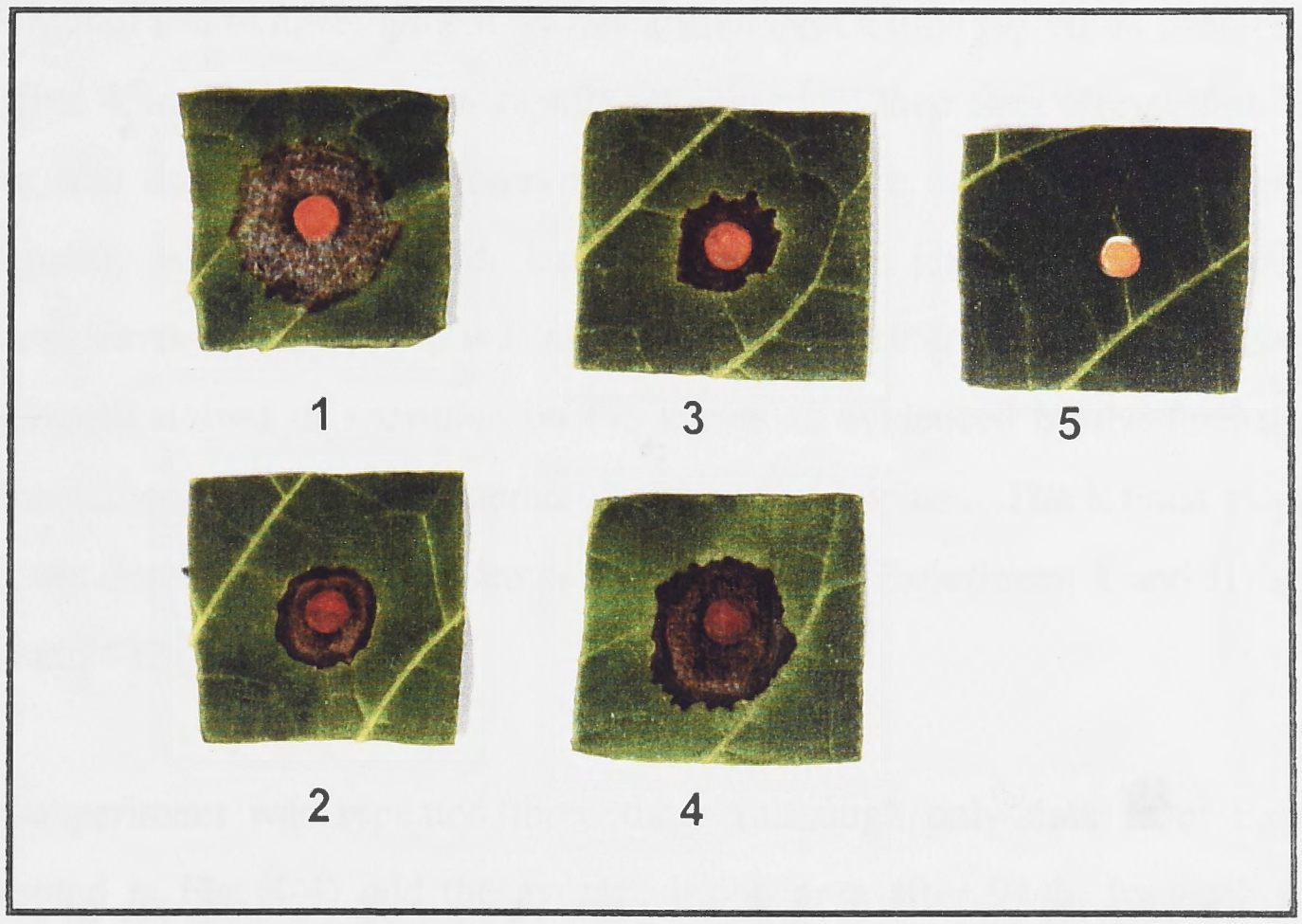
4.3 Results

For initial experiments the leaf disk bioassay was used as a means to determine whether *B. cinerea* lesions were smaller on transgenic leaves expressing Na-PI or β-HTH. It was necessary to ensure that the bioassay was conclusive as well as reliable. However, additional experiments with leaves on whole plants were performed to provide further evidence that transgenic plants containing Na-PI, β-HTH or both genes in combination could inhibit the growth of *B. cinerea.* The results from these experiments are also presented (section 4.3.2). Finally, the culmination of the leaf disk and first whole plant experiments, was a whole plant experiment using second generation tobacco plants derived from homozygous lines. This final experiment was aimed at confirming that the higher levels of Na-PI and β-HTH in these T_2 plants were proportionately more effective in inhibiting *B. cinerea* growth as well as demonstrating that the genes remain biologically active in subsequent generations (section 4.3.3).

4.3.1 *Botrytis cinerea* leaf disk bioassays

Brown lesions caused by *Botrytis cinerea* were visible around wounds in tobacco leaves in all experiments. After four days, the brown necrotic lesion was largest in control leaves compared to those leaves which had been transformed with Na-PI or β-HTH, or both genes together (Fig. 4-4A). No lesions developed on control tobacco leaves which had been infected with a sterile V8 agar plug. Lesions were mostly circular, although irregular shapes were also observed. Lesions appeared to consist of two rings: an outer 'water-soaked' zone, which was lighter in colour contained macerated leaf tissue without fungal hyphae, and an inner necrotic zone, which was dark brown with fungal hyphae growing on the surface (see Fig. 4-4A; Fig. 4.7 C and D). Only the dark brown necrotic zone contained *B. cinerea,* as evidenced by sterilising sections of the leaf, plating on V8 agar and observing hyphal growth after three days. Under these conditions, *B. cinerea* hyphae did not emerge from water soaked lesions, nor as expected, from the uninfected area of leaf (data not shown).

A



B

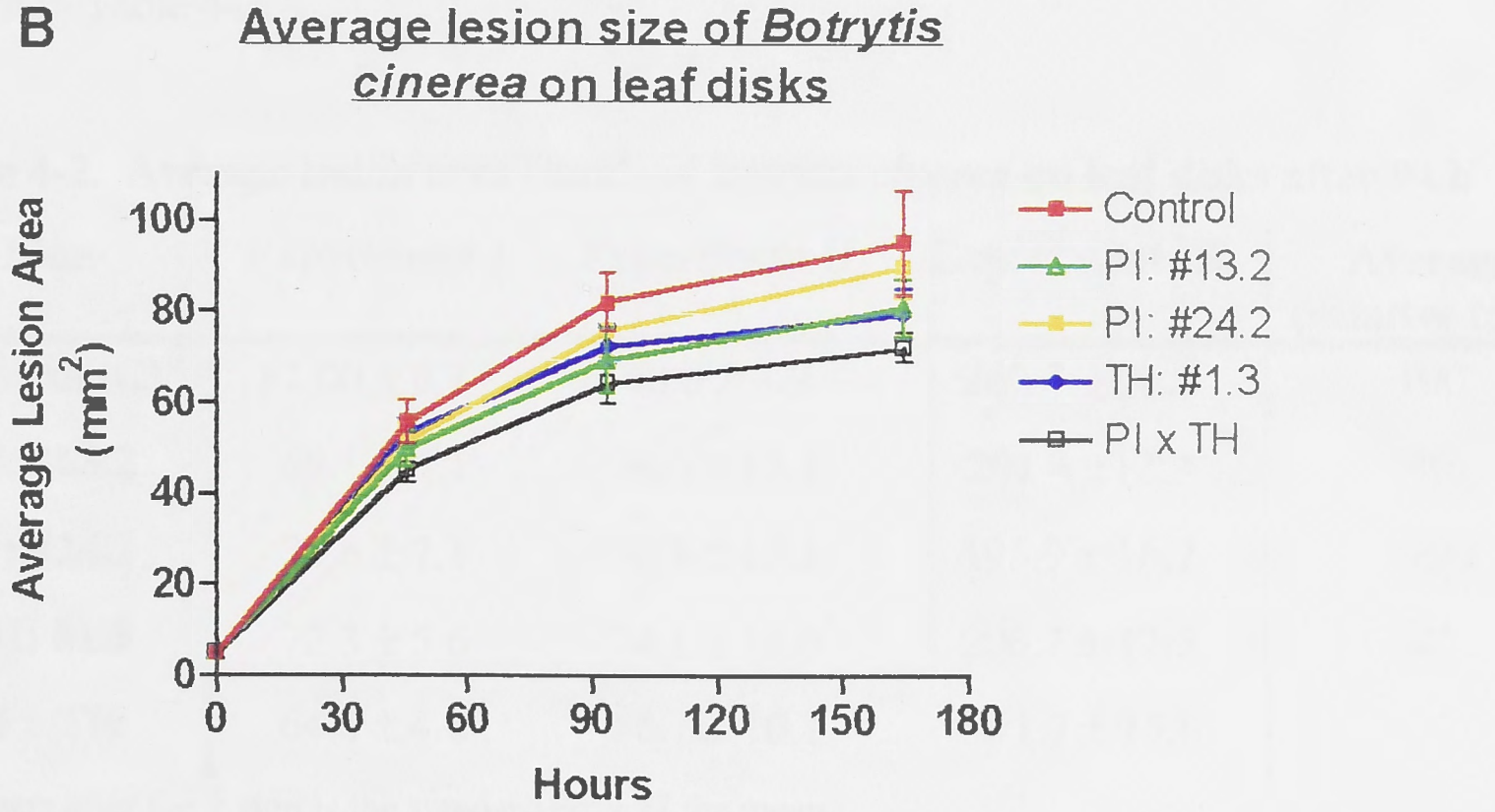


Fig. 4-4 Average lesion area of *Botrytis cinerea* on leaf disks (data from Experiment I)

A. *B. cinerea* growing on leaf disks: 1 is the control (*bar* alone); 2 is TH: #1.3; 3 is PI x TH; 4 is PI: #24.2 and 5 is a control leaf infected only with V8 agar.

B. Growth of *B. cinerea* lesions from 0-165h. Each data point is the average of nine lesion measurements. Error bars represent the standard error of the mean.

The rate of lesion development was measured over a time period of 165h (Fig. 4-4B). In the first 45h, the lesions grew rapidly attaining half their final size in that time. At 45h, there was no significant difference between the size of the lesions, regardless of leaf treatment. After a further 45h, lesions on transgenic leaves were smaller than those on control leaves and this trend was exaggerated after 165h. After approximately 90-120h, *B. cinerea* started to sporulate on the leaves as evidenced by the formation of darkly pigmented conidiophores clustered on the end of hyphae. The lesions stopped growing after six days at the warmer temperature of 27°C (Experiment I and II) and after four days at 24°C (Experiment III).

The experiment was repeated three times (although only data from Experiment I is presented in Fig. 4-4) and the average lesion area after 94 h, for each experiment is shown in Table 4-2.

Table 4-2. Average lesion area (mm^2) of *Botrytis cinerea* on leaf disks after 94 h

Line	Experiment I	Experiment II	Experiment III	Average (relative to C)
Control (C)	82.00 ± 6.3*	90.0 ± 4.9	260.4 ± 20.2	100
PI: #13.2	69.1 ± 6.1	76.6 ± 13.1	201.4 ±12.3	80
PI: #24.2	75.6 ± 7.1	70.8 ± 13.2	195.9 ± 16.7	79
TH: #1.3	72.3 ± 5.6	74.1 ± 16.0	206.7 ± 17.3	83
PI x TH	64.0 ± 4.8	56.7 ± 10.1	191.7 ± 15.6	72

*numbers after the ± sign is the standard error of the mean

In all three experiments, the areas of *B. cinerea* lesions were largest on control tobacco, and smallest on tobacco leaves containing both Na-PI and β-HTH. In general, the order of lesions from largest to smallest was:

Control > PI: #13.2 > TH: #1.3 > PI: #24.2 > PI x TH

Within the error margins, the outcome of the three experiments was consistent. In experiment III, however, regardless of leaf material, the lesions were nearly three times

the size of those in the two previous experiments. The most likely explanation was the different temperature regime, in the third experiment, where infected leaf pieces were incubated at 24°C, instead of 27°C.

The difference between the average size of lesions growing on control leaves, was compared to the average size of lesions on transgenic leaves (Table 4-3). The student's *t*-Test was performed on a number of pairwise comparisons to evaluate if there was any significant difference between the lesion areas. A summary of these comparisons is given below in Table 4-3.

Table 4-3. Two-way comparisons of average lesion area on leaf disks

Comparison	Lesion area difference	*t*-Test result ($\upsilon = 8$)	Significant at P< 0.05?
Control vs PI: #13.2	19.8%	2.72	**YES**
Control vs PI: #24.2	20.8%*	2.44	**YES**
Control vs TH: #1.3	18.3%	2.04	**YES**
Control vs PI x TH	27.8%	3.82	**YES**
PI: #13.2 vs PI: #24.2	1.3%	0.12	NO
PI: #13.2 vs TH: #1.3	1.8%	0.16	NO
PI: #24.2 vs TH: #1.3	3.2%	0.28	NO
PI: #13.2 vs PI x TH	9.9%	1.09	NO
PI: #24.2 vs PI x TH	8.7%	0.81	NO
TH: #1.3 vs PI x TH	11.5%	1.05	NO

*All percentage lesion area differences are reductions

B. cinerea lesions were about 20% smaller on transgenic tobacco containing Na-PI and about 18% smaller on transgenic tobacco transformed with β-HTH. Leaf disks from plants which contained both Na-PI and β-HTH exhibited lesions which were nearly 28% smaller, when compared to lesions on control leaves. The result from the *t*-Test indicated that lesions on transgenic leaves were significantly smaller ($P < 0.05$). This meant there was only a 5% possibility of these results occurring by chance alone.

When the size of the lesions was compared between the two lines of tobacco transformed with Na-PI and the line transformed with β-HTH, the *t*-Test indicated that for the three possible pairwise comparisons, the lesions were not significantly different in size (Table 4-3). This indicated that neither Na-PI or β-HTH were more effective in reducing lesion size.

When Na-PI lines #13.2 and #24.2 were compared to the line containing both Na-PI and β-HTH, there was no statistical difference in lesion area. This implied that the addition of β-HTH to transgenic plants which already contained Na-PI, did not significantly reduce the size of lesions caused by *B. cinerea.* Similarly, there was no statistical difference when line #1.3 containing β-HTH was compared to the double transgenic line indicating that the addition of Na-PI to transgenic plants which contained β-HTH, did not result in significantly smaller lesions, when compared to plants that contained only β-HTH.

An essential part of the analysis was to estimate if any of the variations in the size of lesions was due to either block differences (e.g. 1, 2 and 3), leaf differences (e.g. A, B, and C), or box effects (e.g. 1-9). To determine the contribution of each of these factors, the data from Experiment I was subject to an analysis of variance (ANOVA) using GENSTAT® (Table 4-4).

Table 4-4. Summary of analysis of variance for leaf disk bioassay (Experiment I)

Source of Variation	Degrees of freedom (υ)	F-statistic	P value	Significant
Treatment*	4, 14	3.37	0.039	**YES**
Block (e.g. 1-3)	2, 14	2.00	0.172	NO
Leaf (e.g. A-C)	2, 40	1.53	0.229	NO
Box (e.g. 1-9)	4, 26	1.33	0.279	NO

*treatments were control, PI: #13, PI: #24, TH: #1 and PI x TH

The ANOVA indicated that there was a significant difference in lesion size due to different leaf treatments. There were no differences due to block location, indicating that lesion size was independent of whether the plant was located in block 1, 2 or 3. Likewise, leaf effects were insignificant, or in other words, differences in lesion size were not attributable to the size of the different leaves A, B or C. Finally, regardless of box location, the size of *B. cinerea* lesions was not significantly affected. Therefore, it appeared that the experimental design was statistically robust.

In summary, from the experiments using leaf disks, it appeared that transgenic lines containing either the PI from *Nicotiana alata* or β-hordothionin from barley endosperm inhibited the growth of *B. cinerea.* Plants containing both genes also significantly reduced the size of lesions caused by *B. cinerea*, as expected. However, it appeared that tobacco containing both genes was only marginally more resistant than tobacco containing either gene alone. As the experiment with detached leaves showed that smaller fungal lesions were attributable to Na-PI and β-HTH, the next stage was to assess the performance of *B. cinerea* on whole plants.

4.3.2 *Botrytis cinerea* bioassays using whole plants

4.3.2.1 Experiments with T_1 plants

B. cinerea lesions on control leaves were generally larger than those on transgenic leaves (Fig. 4-5) while leaves which were inoculated with sterile V8 agar did not develop lesions (not shown).

In the whole plant bioassays, lesions caused by *B. cinerea* were measured after ~72 h. Representative data (from Experiment III) for the area of lesions on control and transgenic tobacco after inoculation with *B. cinerea* is shown in Fig. 4-6.

Fig. 4-5. Photographs of *Botrytis cinerea* whole plant (T_1) bioassay.
A. Experimental setup in PH1 glasshouse. B. A single transgenic plant with each leaf covered by a plastic bag. C. Tobacco leaves 72h after inoculation with *B. cinerea.* Clockwise from left, control; PI x TH; TH: #1.3 and PI:#24.2. D. Lesions on control and PI x TH leaves. Lesions on PI x TH plants were significantly smaller than on control leaves ($P < 0.05$).

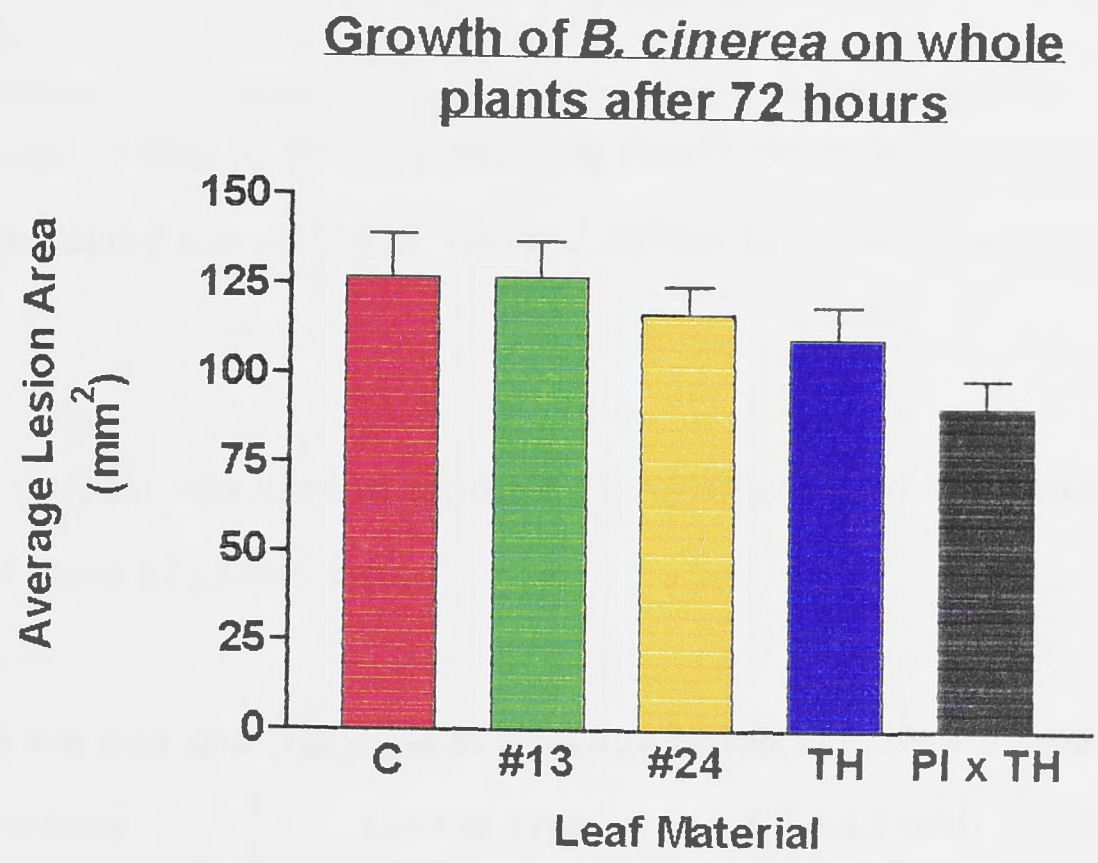


Fig. 4-6. Histogram of lesion area of *Botrytis cinerea* on leaves of T_1 plants.
Average leaf area 72h after inoculation with V8 agar containing *B. cinerea* hyphae. Area was estimated from the diameter of the necrotic lesion which formed around a small wound in the leaf surface. Each coloured bar is the average of 36 lesions and error bars are the standard error of the mean. The label C refers to control plants, #13 and #24 are T_1 transgenic tobacco plants containing Na-PI. The labels TH and PI x TH refer to transgenic plants containing β-hordothionin alone, or in combination with Na-PI, respectively. Differences in lesion area were significant only on PI x TH plants, relative to controls.

The experiment infecting whole tobacco plants with *B. cinerea* was repeated three times. Average lesion data from all experiments is presented in Table 4-5.

Table 4-5. Average lesion area (mm^2) of *B. cinerea* on T_1 plants after 72 h

Line	Experiment I	Experiment II	Experiment III	Average (relative to C)
Control (C)	77.0 ± 14.3	141.2 ± 8.3	154.6 ± 14.9	100
PI: #13.2	82.2 ± 5.5	139.9 ± 7.6	157.3 ± 16.6	102
PI: #24.2	78.9 ± 6.8	115.1 ± 5.9	154.5 ± 11.8	94
TH: #1.3	68.1 ± 3.0	116.5 ± 7.2	143.2 ± 17.3	88
PI x TH	51.1 ± 10.3	110.7 ± 5.7	109.3 ± 8.0	73

In all whole plant experiments, lesions on transgenic tobacco transformed with only β-HTH or with both β-HTH and Na-PI were smaller than those on control plants containing the selectable marker gene (Fig. 4-5, 4-6 and Table 4-6) and only the latter

was significant. Tobacco plants containing Na-PI (lines #13.2 and #24.2) were not as effective in reducing the size of *B. cinerea* lesions as results from the leaf disk bioassay suggested.

The student's *t*-Test was used to estimate the significance of differences of lesion size on different leaf material (Table 4-6).

Table 4-6. Two-way comparisons of average lesion areas on whole plants (T_1)

Comparison	Lesion area difference	*t*-Test result ($\upsilon = 35$)	Significant at $P < 0.05$?
Control vs PI: #13.2	+ 1.8%	0.18	NO
Control vs PI: #24.2	6.5%*	0.65	NO
Control vs TH: #1.3	12.1%	1.20	NO
Control vs PI x TH	27.3%	2.71	**YES**
PI: #13.2 vs PI: #24.2	8.2%	1.04	NO
PI: #13.2 vs TH: #1.3	13.6%	1.74	NO
PI: #24.2 vs TH: #1.3	5.9%	0.75	NO
PI: #13.2 vs PI x TH	28.5%	3.64	**YES**
PI: #24.2 vs PI x TH	22.2%	3.14	**YES**
TH: #1.3 vs PI x TH	17.3%	2.05	**YES**

*Percentage lesion area differences are reductions unless otherwise stated

As shown for the leaf disk bioassays, lesion areas on either of the transgenic plants expressing only one defence gene were similar, so Na-PI was not more effective than β-HTH at protecting tobacco against *B. cinerea.*

For the reasons described in the leaf disk bioassay, one experiment was fully analysed to determine if the random errors, (block 1-3; leaf A-C; lesion 1-4), contributed to the observed variation between treatments. The ANOVA results indicated that none of these factors had a significant effect on lesion size (data not shown).

In summary, the results from these whole plant bioassays with T_1 plants showed that only transgenic plants containing both Na-PI and β-HTH were more protected against infection by *B. cinerea.* Contrary to the leaf disk bioassay, lesions on tobacco containing either Na-PI or β-HTH alone were not significantly smaller than those on control plants. It is possible that there is a gene-dosage effect, and as T_1 populations of transgenics plants would contain plants which were both hetero- and homozygous, the bioassay was used to assess the performance of T_2 individuals derived from a homozygous line, or in the case of PI x TH; T_3 individuals which were homozygous for both genes.

4.3.2.1 Experiments with T_2 plants

As for the previous whole plant bioassay, *B. cinerea* lesions were much smaller when grown on transgenic tobacco homozygous for Na-PI or β-HTH and on plants containing both β-HTH and Na-PI, relative to lesions on control plants (Fig. 4-7A). A histogram of the average area of lesions formed on the four plant lines is shown in Fig. 4-7. As results from the three whole plant bioassays using T_1 plants were reproducible, the *B. cinerea* bioassay with T_2 plants was performed only once because the data would provide a reliable indication of the gene-fungal interaction.

Lesions developing on control leaves were the largest, while lesions on transgenic tobacco containing 0.38% Na-PI (of total soluble protein (TSP)) were marginally smaller. The size of lesions on transgenic tobacco with Na-PI (0.5% TSP) and β-HTH, however were about half the size of control lesions. To effectively compare the lesions, pair-wise comparisons were performed on the four leaf treatments to determine if the observations were statistically significant (Table 4-7).

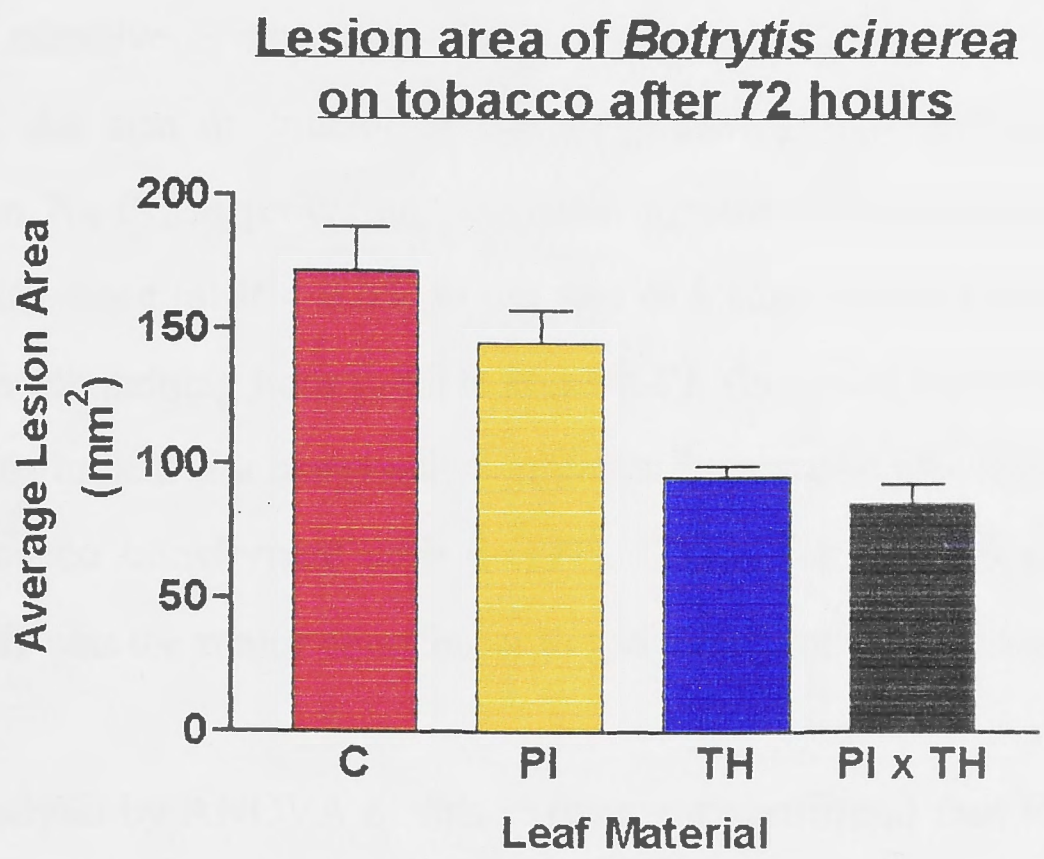


Fig. 4-7 Histogram of lesion area of *B. cinerea* on T_2 plants
Necrotic lesions were measured on tobacco leaves, 72h after inoculating with *B. cinerea.* Each coloured bar is the average of 36 lesions while the error bars represent the standard error of the mean. Tobacco control (C) plants, or transgenic Na-PI (PI) or β-HTH (TH); or β-HTH in combination with Na-PI (PI x TH), were significantly more protected from *B. cinerea,* relative to untransformed tobacco plants.

Table 4-7. Two-way comparisons of average lesion areas on whole plants (T_2)

Comparison	Lesion area difference	*t*-Test result	Significant at P< 0.05?
Control vs PI: #24.2	15.7%*	2.14	YES
Control vs TH: #1.3	44.7%	4.73	YES
Control vs PI x TH	50.5%	5.34	YES
PI: #24.2 vs TH: #1.3	34.4%	3.94	YES
PI: #24.2 vs PI x TH	41.%	4.73	YES
TH: #1.3 vs PI x TH	10.4%	1.33	NO

*Lesion area differences are reductions

Lesions were significantly smaller on all transgenic plants, relative to control tobacco (Fig. 4-7; Table 4-7). Furthermore, the *t*-Test showed that the probability of this outcome by chance, is less than 5%. Na-PI had the effect of reducing lesion area by nearly 16%. Transgenic tobacco containing β-HTH alone, or β-HTH in combination with Na-PI,

proved very effective in protecting tobacco against *B. cinerea,* with lesions being ~45% or ~51% of the size of control lesions, respectively. β-HTH appeared to be more effective than Na-PI, in providing resistance against *B. cinerea* because there was no significant difference (at $P < 0.05$) in the size of lesions on either tobacco containing β-HTH alone or containing both β-HTH and Na-PI, (however the result was significant at $P = 0.1$). This indicated a marginally significant increase in protection by the addition of Na-PI to tobacco transformed with β-HTH. Thus, for tobacco plants containing two genes, β-HTH was the major contributor to the joint protective effect of the two genes.

Statistical analysis by ANOVA of this experiment confirmed that the variation between blocks (1-3); leaves (A-C) and lesions (1-4) did not significantly contribute to the variation observed between the four different plant lines (data not shown).

B. cinerea hyphae grown on control or transgenic material was examined by light microscopy to identify any hyphal characteristics which differed for *B. cinerea* growing on either the control or PI x TH. On the control leaf, *B. cinerea* were white and at the time of photography, conidiospores were prolific on hyphal stalks (Fig. 4-8C). The brown necrotic lesion was surrounded by a 3 mm ring of tissue which appeared to have been damaged by water-soaking. In control tissue, this zone of damage appeared to move in advance of hyphal penetration, but it was rarely seen in the lesions which formed on transgenic leaves (Fig. 4-8D). At the very periphery of the lesion formed on control leaves, there was a dark brown ring at the border of damaged and non-damaged tissue (Fig. 4-8C). Lesions growing on transgenic tissue looked different (Fig. 4-8D). The *B. cinerea* hyphae were grey-white and covered a smaller area, though were often more dense than those growing on control leaves. Occasionally there was a zone of water-marked damage, although, generally it was less defined. At the border between infected and non-infected tissue there was always a very distinct grey zone, which appeared dry - it is possible that this area contained plant cells which had dehydrated. Therefore, the main difference of the appearance of *B. cinerea* between controls and transgenics is the

size of the watersoaked zone, being larger, and dark brown in controls and small, grey and dry in transgenics.

The second approach was to clear the leaf tissue surrounding the lesion and to use lactophenol-cotton blue (section 4.2.2.2) to observe the hyphae penetrating uninfected tissue. *B. cinerea* mycelium were stained blue (Fig. 4-8B). At this level of microscopy it there appeared to be no morphological characters which distinguished hyphae growing on control leaves from hyphae penetrating transgenic leaves. Scanning electron microscopy was used to resolve detail on the hyphal structure. On control leaves, *B. cinerea* hyphae were long and ribbon-like and appeared to spread out over the entire lesion area (Fig. 4-8E). Damaged leaf tissue appeared flat with the definition of a raised leaf surface being lost. Trichomes (leaf hairs) were destroyed and flattened. On transgenic PI x TH leaves, *B. cinerea* hyphae appeared slightly thicker and shorter with many convolutions. Hyphae grew more densely and did not grow outwards in the same way as was observed on control leaves. In addition, SEM of transgenic PI x TH leaves, revealed an obvious zone of tissue in between the infected and non-infected leaf where *B. cinerea* did not grow.

In conclusion, it appeared that transgenic plants containing either Na-PI, β-HTH or both genes together, were more protected against *B. cinerea* than the untransformed control plants. As β-HTH was so effective in protecting tobacco against *B. cinerea* the next strategy was to assess if transgenic plants containing β-HTH were more protected against the pathogenic bacterium *Pseudomonas solanacearum.*

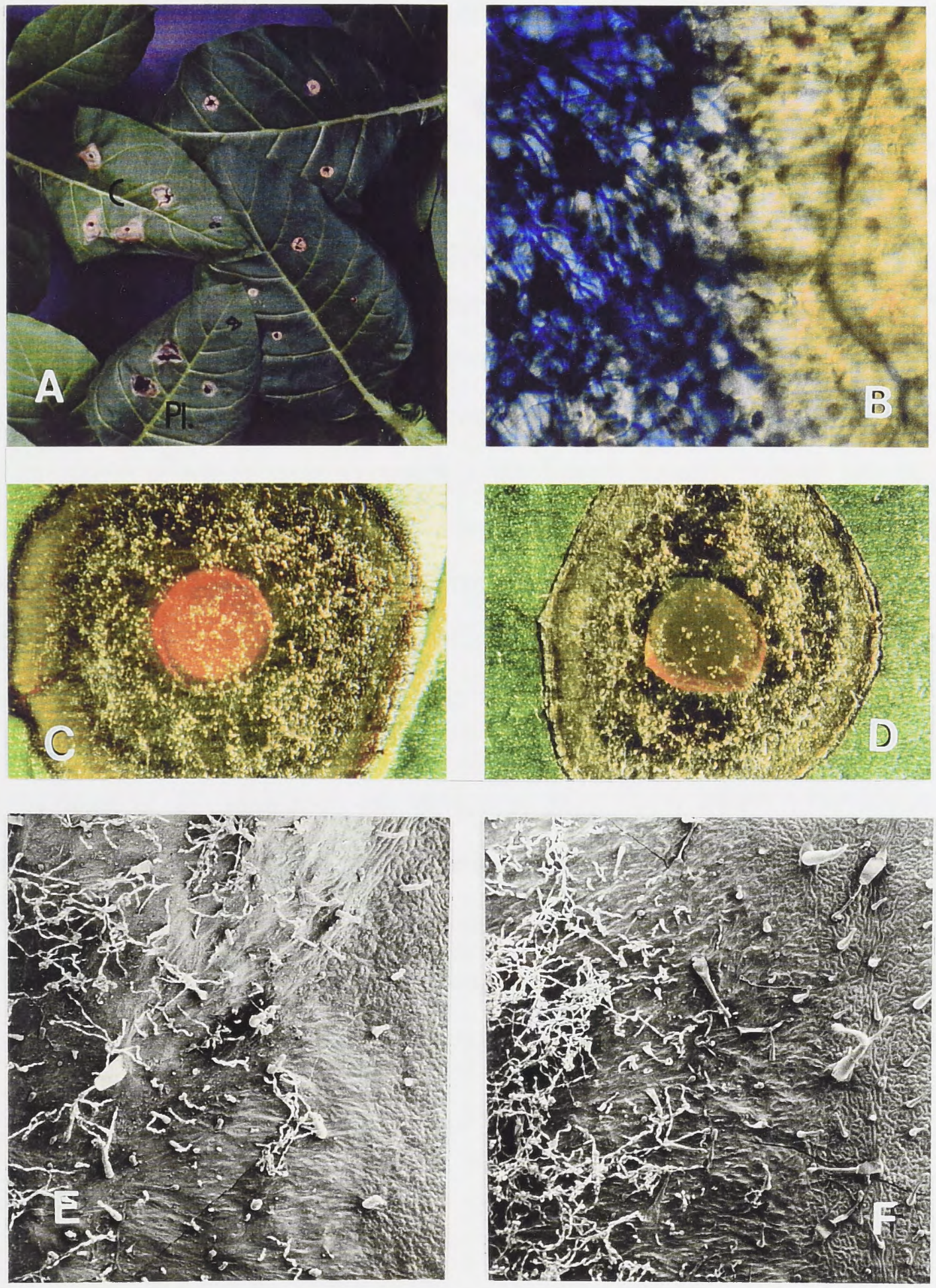


Fig. 4-8 Appearance of *Botrytis cinerea* and lesions on leaves of T_2 plants.
A. Leaves from tobacco plants infected with *B. cinerea.* Clockwise from left: control; PI x TH; TH:#1.3; and PI: #24.2. B. *B. cinerea* stained with lactophenol-cotton blue. Hyphae are penetrating previously uninfected tissue as seen through light microscope (100x). C. *B. cinerea* on control tissue, using stereo microscope (6x). D. *B. cinerea* on PI x TH transgenic tissue using stereo microscope (6x). E. Scanning electron micrograph (SEM) of *B. cinerea* infecting an untransformed leaf. F. SEM of *B. cinerea* infecting a leaf from tobacco containing PI and TH.

4.3.3 *Pseudomonas solanacearum* bioassay

As these experiments were performed in the later stages of this project, only T_2 or T_3 (PI x TH) plants were used. Wilted leaves were apparent on tobacco plants three days after injecting the soil surrounding the roots of seedlings with a dilute inoculum of *P. solanacearum.* After seven days, symptoms were more severe on control tobacco seedlings than on transgenic plants expressing β-HTH or plants expressing Na-PI and β-HTH (Fig. 4-9). Whether the whole plant was dead, or only one leaf was wilted, it was scored as possessing symptoms characteristic of infection by *P. solanacearum.* After seven days, plants which were completely dead were mainly control plants, while in general, transgenic plants had only minor symptoms. None of the seedlings which had been sham-inoculated with sterile liquid growth media developed symptoms (P. Hughes; pers. comm.). The percentage of plants which became infected by *P. solanacearum* after seven days was plotted as a histogram (Fig. 4-10).

The infectivity of *P. solanacearum* was very high, with ~94% of control tobacco seedlings showing wilting symptoms, after only seven days. By comparison, 75% of β-HTH transformants had symptoms, while only 64% of tobacco with both Na-PI and β-HTH became infected with *P. solanacearum.* The difference between percentage infectivity for the three plant lines was analysed using the student's *t*-Test. The results of the comparisons are shown in Table 4-8.

Fig. 4-9. *P. solanacearum* bioassay.
A. Growth cabinet with boxes of tobacco seedlings. There were 40 boxes of each tobacco line (Control, TH: #1.3 and PI x TH) and each box contained four plants. B. Five week old tobacco seedlings, seven days after inoculation with *P. solanacearum.* From left; control plants, tobacco seedlings with β-HTH; double transgenics (PI x TH).

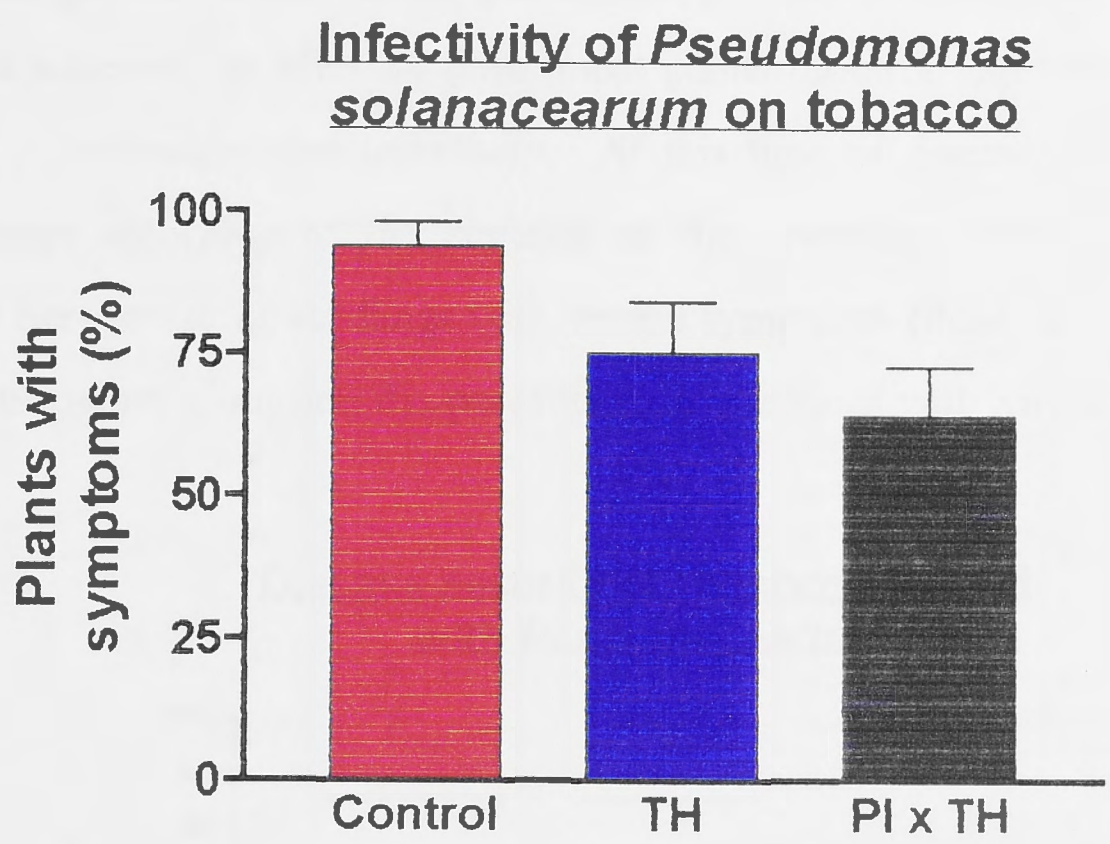


Fig. 4-10. Infectivity of *P. solanacearum* on tobacco
Histogram of the percentage of tobacco seedlings which showed disease symptoms, seven days after inoculating roots with *P. solanacearum*. The coloured bars are the average rate of infection for 40 boxes, and the vertical bars are the standard error of the mean for each treatment. Tobacco containing either β-HTH (TH); or β-HTH in combination with Na-PI (PI x TH), were significantly more protected from *P. solanacearum*, relative to control tobacco plants.

Table 4-8. Two-way comparisons of *P. solanacearum* infectivity on tobacco (T_2)

Comparison	*t*-Test result ($\upsilon = 39$)	Significant at $P < 0.05$?
Control vs TH: #1.3	2.07	YES
Control vs PI x TH	3.51	YES
TH: #1.3 vs PI x TH	1.18	NO

The difference in percentage infectivity of control tobacco versus transgenic tobacco containing β-HTH was significant. Likewise, tobacco plants transformed with both Na-PI and β-HTH were significantly more protected against *P. solanacearum* infection than control plants transformed with only the selectable marker gene. Because the *t*-Test demonstrated that there was no significant difference between the plants transformed with β-HTH and those transformed with Na-PI and β-HTH, this indicated that Na-PI did not confer additional resistance against *P. solanacearum*. The results from the *t*-Test were consistent for both Experiment I and II.

Tobacco seedlings were under severe pathogenic pressure because the boxes had been sealed without watering, so after 14 days, most plants (control and transgenics) showed symptoms of *P. solanacearum* infectivity. At this time, a disease index was used to classify seedlings according to the severity of the symptoms they exhibited. As a summary, the percentage of seedlings with severe symptoms (dead or very wilted) were plotted on a histogram alongside the percentage of seedlings with virtually no symptoms (Fig. 4-11).

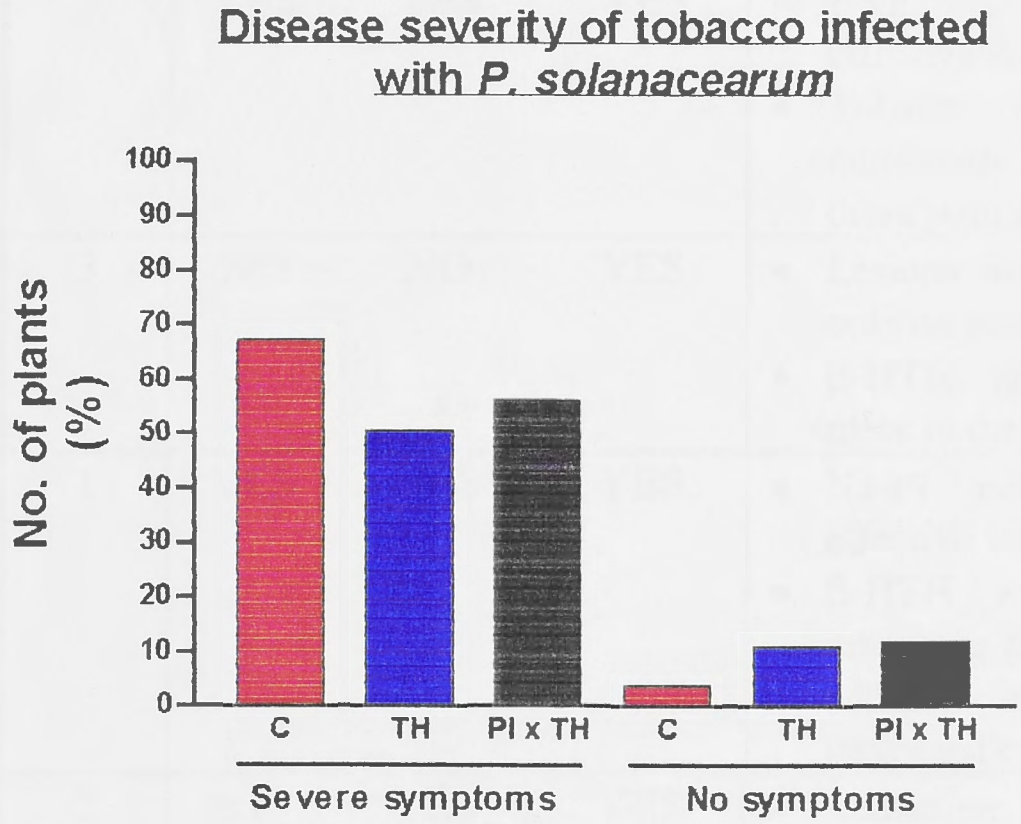


Fig. 4-11 Disease severity of *P. solanacearum* on tobacco seedlings.
Tobacco were classified as having severe or no symptoms, 14 days after inoculation with *P. solanacearum*. More control (C) tobacco seedlings had severe symptoms than transgenic tobacco containing β-HTH (TH) or both β-HTH and Na-PI (PI x TH) in combination.

After 14 days, there were more control plants with severe symptoms compared to plants transformed with β-HTH or transformed with both Na-PI and β-HTH. Moreover, there were over four-fold more transgenic plants with no disease symptoms, when compared to control plants.

4.4 Discussion

Results from a series of experiments assessing tobacco transformed with Na-PI and β-HTH, or both genes in combination, for improved resistance to *B. cinerea* and *P. solanacearum* have been presented. The major conclusions are summarised in Table 4-9.

Table 4-9. Effects of Na-PI and β-HTH on *B. cinerea* and *P. solanacearum*

Bioassay	No. of Expts	Effect significantly different to controls?			Comments
		Na-PI	β-HTH	PI x TH	
***B. cinerea* Leaf disk**	3	YES	YES	YES	• Both Na-PI and β-HTH were effective in reducing lesion area. • Tobacco with two genes, were marginally more protected than those with a single gene
Whole plant (T_1)	3	NO	NO	YES	• Lesions were significantly smaller only on tobacco with both genes. • β-HTH appeared to contribute more to the protective effect.
Whole plant (T_2)	1	YES	YES	YES	• Na-PI and β-HTH were both effective in reducing lesion area. • β-HTH was more effective in inhibiting fungal growth, although addition of Na-PI marginally increased resistance to *B. cinerea.*
***Pseudomonas solanacearum* (seedlings)**	2	n.t*	YES	YES	• Reduction in disease severity was attributed to the presence of β-HTH because Na-PI did not provide any additional protection.

*n.t - not tested

4.4.1 Inhibition of fungal growth by Na-PI

Na-PI significantly reduced the growth of *B. cinerea* in both the leaf disk bioassay and the T_2 whole plant bioassay (Table 4-9). One explanation as to why Na-PI was more effective in leaf disk bioassays, than in T_1 whole plant bioassays, is that wounding may have induced other plant defence molecules (Green and Ryan, 1972; Plunkett *et al.*, 1982) that provide an additive effect to Na-PI - a response not attainable for control plants. In intact T_1 plants, the wounding response would not have been so prevalent thus not triggering the production of endogenous PIs. An alternative explanation is that the level of Na-PI was higher in the leaves used in leaf disk bioassays, or that the leaves were slightly younger. For example, the position of leaves on the plant influences the level of Na-PI, yet as the level of Na-PI was not determined in every leaf, this also could have been a factor which contributed to the variation between the leaf disk and whole plant

bioassays, even though both experiments used T_1 populations. It is possible that leaves for disk assays came from three tobacco plants which were all homozygous. In homozygous plants, the level of Na-PI would be about 0.38%, which is higher than in heterozygous individuals (0.28%). Conversely, in T_1 whole plant experiments, perhaps all or two thirds of the plants were heterozygous, and consequently the protective effect was proportional to the level of Na-PI. In a uniform population of T_2 tobacco plants, derived from the homozygous line #24.2, the effect of Na-PI significantly inhibited the growth of *B. cinerea.* The outcome of the T_2 experiments is likely to be the most valid result, as the level of Na-PI in the three chosen plants was known.

Although there has been much research aimed at exploiting PIs as potential fungicidal agents, little is known about the mechanism by which PIs retard fungal growth. One possibility is that PIs inhibit fungal proteases, which would otherwise be essential for growth (Ryan, 1990). For example, in colonising its host, *B. cinerea* produces a wide range of toxins, cell wall degrading enzymes, lipases and proteases (Coley-Smith *et al.,* 1980; Brown and Adikaram, 1983). The first evidence for a role of PIs in protection from fungal pathogens was the discovery that plant PIs form complexes with proteinases of microbial origin. For example, trypsin inhibitors from kidney bean seeds inhibit the extracellular serine-like protease secreted by *Colletotrichum lindemuthianum* (Mosolov *et al.,* 1979). Another example is that the proteinases of *Streptomyces griseus* are inhibited by potato chymotrypsin inhibitor (Ryan, 1966).

Moreover, in most cases studied to date, no plant proteinases are endogenous substrates for plant proteinase inhibitors (Ryan and Walker-Simmons, 1981). This is also true for the proteinase inhibitor from *Nicotiana alata.* These trypsin and chymotrypsin inhibitors are expressed to high levels in the stigma (Atkinson *et al.,* 1993a), yet this organ does not contain either trypsin or chymotrypsin. Na-PI inhibits serine proteases from insect gut extracts (Heath, 1995; Heath *et al.,* 1997), and has shown inhibitory activity *in vitro* towards micro-organisms (Dunse, 1992; M. Muskins, pers. comm.). Na-PI was strongly inhibitory to *B. cinerea* and *Phytophthora nicotianae* as measured by the zone of growth inhibition in agar plates. Similarly, soybean and potato trypsin inhibitors strongly

inhibited *B. cinerea* and *P. nicotianae*, as well as *Chalara elegans* (Dunse, 1992). In addition, Na-PI was able to inhibit the germination of *B. cinerea in vitro,* particularly in the first 25 hours of growth (M. Muskins, pers. comm). In light of the *in vitro* evidence described above, the demonstration that tobacco transformed with Na-PI inhibited the growth of *B. cinerea in vivo,* both extends and supports the findings of Dunse (1992) and M. Muskins. It appears therefore, that one biological role of Na-PI, *in vivo,* could be to contribute to protecting *N. alata* stigmas from colonisation by fungal pathogens.

Although not tested here, there is scope for the use of PIs for the protection of plants against bacteria. As do fungi, bacteria secrete proteinases which are employed during microbial invasion of plants (Reddy *et al.,* 1966; Porter, 1966), usually for the hydrolysis of proteins in and between cell walls (Grinzburg, 1961; Newcomb, 1963). For example, *Erwinia* species produce numerous pectic enzymes, proteinases and hydrolases in order to macerate host tissue, in advance of invasion (Moran *et al.,* 1968; Mount *et al.,* 1970). Moreover, bacterial proteases from *Bacillus subtilis* and *Streptomyces griseus* were inhibited by potato chymotrypsin inhibitor (Ryan, 1966). Interestingly, out of the PIs tested in the diffusion assay, only the soybean trypsin inhibitor conclusively inhibited *P. solanacearum,* but this bacterium was only weakly inhibited by Na-PI (Dunse, 1992).

4.4.2 Antimicrobial activity of β-HTH

Expression of the barley β-hordothionin conferred enhanced resistance to the fungal pathogen *B. cinerea* (in two out of three experiments) and to the bacterial pathogen *P. solanacearum* (Table 4-9). Overall, β-HTH appeared more effective in reducing *B. cinerea* lesion area, than Na-PI. Although there is no evidence presented here, it is possible that tobacco plants express β-HTH to higher levels in leaves than Na-PI. Alternatively, *B. cinerea* may be more sensitive to the toxic effect of thionins than proteinase inhibitory activity of Na-PI. Furthermore, the subcellular location of Na-PI and β-HTH would determine the extent of the interaction between these defence molecules and *B. cinerea*. For example, because Na-PI has a putative vacuolar targeting sequence (Nielsen *et al.,* 1996), it is thought that Na-PI in leaves of transgenic plants would be targeted to the vacuole (M. Anderson, pers. comm.). Thus, the intracellular

location of Na-PI may mean that cell lysis must occur for the fungus to be exposed to its inhibitory effects. In transgenic plants β-HTH may also accumulate intracellularly, as *in vivo* it accumulates in the electron-dense spheroides in the periphery of the protein bodies (Carmona *et al.,* 1993a). Perhaps if β-HTH was in a location that was more accessible to fungal contact, this could explain the apparent differences in effect of the two genes on *B. cinerea.*

As for PIs (section 4.4.1) the hypothesis of a defence role for thionins was based on their antimicrobial activity towards plant pathogens *in vitro* (Bohlmann *et al.,* 1988; Florack *et al.,* 1993; Molina *et al.,* 1993; Terras *et al.,* 1993a) and their induction in response to biotic and abiotic stress (Bohlmann *et al.,* 1988; Fisher *et al.,* 1989). Increased resistance of tobacco against *Pseudomonas syringae* pv. *tabaci* has previously been achieved through the transgenic expression of α-hordothionin (α-HTH) from barley endosperm (Carmona *et al.,* 1993b). However, this antifungal effect may be strain specific, as transgenic tobacco containing α-HTH, produced by Florack *et al.,* (1994) were no more tolerant to a different race of *Pseudomonas syringae* pv. *tabaci.* In this work, transgenic tobacco plants expressing β-HTH were significantly more tolerant to *P. solanacearum,* than control plants as measured by development of symptoms. Conversely, tobacco or tomato expressing high levels of α-HTH were not protected against a different strain of *P. solanacearum* (cited in Florack and Stiekema, 1994) and again, this might be explained by differences in bacterial strains. Interestingly, the α-HTH from the aforementioned transgenic tobacco were biologically active, as *in vitro* assays clearly demonstrated they inhibited the growth of *Clavibacter michiganensis* subspecies *michiganensis* (Florack *et al.,* 1994). Another explanation for transgenic tobacco not being more resistant to bacterial infection, could be that α-HTH was localised intracellularly (Florack *et al.,* 1994), while bacteria multiply extracellularly (Isaac, 1992).

Several groups have expressed other plant proteins with antimicrobial activities in transgenic plants. For example, transgenic tobacco and canola expressing bean chitinase

had increased resistance to *Rhizoctonia solani* (Broglie *et al.,* 1991). Likewise, tobacco transformed with barley ribosome inactivating protein were also more protected against *R. solani* (Logemann *et al.,* 1992). Another group of antimicrobial peptides (AMPs), characterised by their abundance of cysteines, include plant defensins (e.g *Rs*-AFP from radish; Broekaert *et al.,* 1995), knottin-type peptides (e.g. *Mj*-AMP from *Mirabilis jalapa;* Cammue *et al.,* 1992), hevein-type peptides (e.g. *Ac*-AMP from *Amaranthus caudatus;* Broekaert *et al.,* 1992) and lipid transfer proteins (Terras *et al.,* 1992b). Recently, the transfer of *Rs*-AFP conferred additional protection to transgenic tobacco, from *Alternaria longipes* (Terras *et al.,* 1995). There are also other cysteine-rich AMPs which may be useful as transferable resistance traits for genetic engineering of crop plants. For example, expression of *Arabidopsis* lipid transfer proteins in transgenic tobacco plants increases their tolerance to *P. solanacearum* (P. Hughes and J. Charity, pers. comm.). The constitutive expression of *Mj*-AMP or *Ac*-AMP in transgenic tobacco, however, did not result in increased protection against infection by *B. cinerea* or *A. longipes* (De Bolle *et al.,* 1996), though extracts from these plants showed inhibitory activity, *in vitro* towards *Fusarium culmorum,* (cited in Cammue *et al.,* 1994) and *B. cinerea* (De Bolle *et al.,* 1996).

Besides transferring genes which encode antimicrobial peptides, an alternative strategy is to engineer plants with an additional enzyme to increase phytoalexin production (e.g. stilbene synthase). This approach has resulted in the production of transgenic tobacco with more resistance to infection by *B. cinerea* (Hain *et al.,* 1993). Currently, new sources of defence genes are being sought and an increasing number of genes encoding antimicrobial peptides are being exploited from species, other than plants. For example, effective disease resistance has also been obtained in transgenic tobacco transformed with a tabtoxin, isolated from *Pseudomonas syringae* pv. *tabaci* (Anzai *et al.,* 1989). Finally, there are several potent antibacterial proteins derived from insects (cecropins) which may find application in molecular breeding of plants for crop protection (Jaynes *et al.,* 1987).

4.4.3 Protective effect of two defence genes

One way to create transgenic crops with effective and durable resistance, is to engineer plants with more than one type of antimicrobial peptide. Theoretically at least, two or more genes in combination should lower the rate of emergence of resistant pathogens. Fungal and bacterial bioassays were used to assess the possible synergism of Na-PI and β-HTH, in combination. In general, the addition of Na-PI to plants already transformed with β-HTH slightly enhanced resistance to *B. cinerea,* but not to *P. solanacearum.* This meant that the effect of the two genes was marginally additive for the fungal pathogen, but not so for bacteria. By comparison, the transformation of other plants with more than one gene has often resulted in the synergistic enhancement of the effect of the genes. For example, when tomato was transformed with a tobacco class I chitinase and a class I β-1,3-glucanase, the genes acted synergistically to enhance resistance to the fungus *Fusarium oxysporum* f.sp. *lycopersici* race 1 (Jongedijk *et al.,* 1995). Similarly, when tobacco was transformed with three genes from barley; chitinase, β-1,3-glucanase and ribosome inactivating protein, the effect was to confer synergistically-enhanced tolerance to infection by *Rhizoctonia solani* (Jach *et al.,* 1995).

A study of the interaction between PIs and thionins in transgenic plants is novel, and to my knowledge, there is no other literature on the transformation of plants with this *combination* of defence genes. There is, however, some *in vitro* evidence that the antifungal activity of wheat thionins could be enhanced by barley trypsin inhibitors (Terras *et al.,* 1993a). In these experiments, the *in vitro* antifungal activity of α-purothionin was enhanced 3- to 20-times if barley '2S-like' trypsin inhibitor was added to the media, depending on the fungus tested. However, when other barley trypsin inhibitors (i.e.; not '2S-like') were used, synergy was not apparent. Interestingly, if α-purothionin was substituted with barley α- or β-hordothionin, there was no synergy with barley trypsin inhibitors, when tested on *Fusarium culmorum* (Terras *et al.,* 1993a). The latter is in agreement with the effect of Na-PI and β-HTH on *B. cinerea* shown in the experiments presented here.

Finally, *B. cinerea* challenged with a combination of Na-PI and β-HTH exhibited minor morphological changes (Fig. 4-7E and F). More significant though was both the appearance and reduction in the size of the water-soaked zone on double transgenic leaves, compared to control leaves. One effect of Na-PI in transgenic plants could be to inhibit the trypsin- and chymotrypsin-like proteases of *B. cinerea* which it uses to macerate leaf tissue, in advance of hyphal penetration. Evidence for this is that the extent of maceration by *B. cinerea* was minimal in transgenic plants, compared to controls. β-HTH may also have contributed to the decrease in the size of the water-soaked zone, perhaps by causing cell membrane damage and intracellular leakage, as has been reported previously (Carrasco *et al.,* 1981; García-Olmedo *et al.,* 1993).

There is increasing evidence that fungi experience morphological changes when challenged with physiological stress. For example, when the pistils of strawberries (which are a rich source of defence molecules), are inoculated with *B. cinerea,* the growth of fungal hyphae appears highly branched and stunted (Bristow *et al.,* 1986). Conversely, in a study of the morphology of *B. cinerea* which had penetrated the stigmas of *Nicotiana alata,* the diameter of hyphae and branching pattern appeared normal (Dunse, 1992). Morphological analysis of fungi affected by cysteine-rich AMPs demonstrated some important differences between some of the AMP classes. *Rs*-AFP, for example, caused thickening of the hyphal branches and the appearance of swollen regions at the hyphal tips (Terras *et al.,* 1993b), while the *Mj*-AMPs or the *Ac*-AMPs do not change the normal mycelial morphology (Cammue *et al.,* 1994). In comparison to *Rs*-AMP which altered hyphal morphology, one of the main effects of β-HTH and Na-PI led to a higher concentration of fungal hyphae in a infected tissue. One explanation for an increase hyphal density is that invasion into surrounding tissue is impeded by Na-PI and β-HTH. It would be interesting to further characterise the nature of the interaction between Na-PI and β-HTH and fungal pathogens.

4.5 Chapter summary

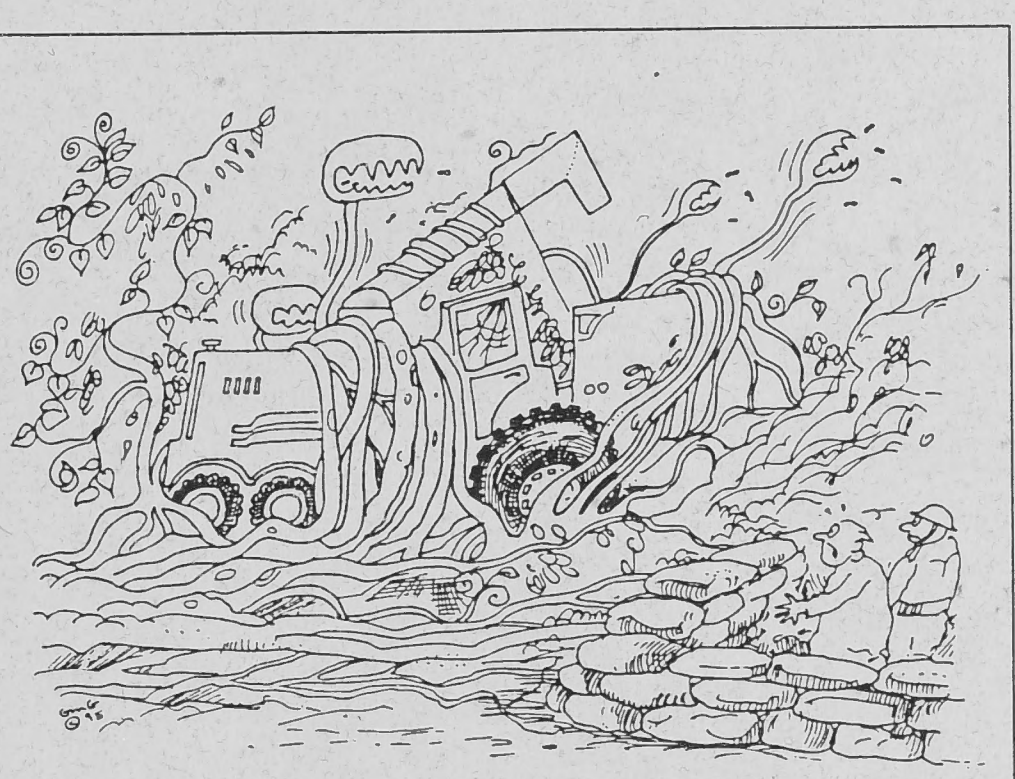
This chapter provided evidence that the proteinase inhibitor from *Nicotiana alata* and the β-hordothionin from barley endosperm should be considered among the suite of antimicrobial peptides available for genetic improvement of crop plants, because tobacco plants expressing these genes were more resistant to important phytopathogenic fungi and bacteria.

Transgenic tobacco containing either Na-PI or β-HTH were significantly more protected from the fungal pathogen, *Botrytis cinerea,* compared to control plants, as indicated by the size of necrotic lesions on leaves. Out of the two genes, β-HTH appeared more effective in reducing fungal lesion size and it is possible that *B. cinerea* was more toxic to this protein. Moreover, transgenic tobacco containing both genes together, were significantly more tolerant to *B. cinerea* than untransformed tobacco. Although the effect of the two genes was additive, the contribution of each gene to the protective effect, however, was not equal. For example, β-HTH provided a major increase in resistance, while the addition of Na-PI only marginally increased fungal tolerance.

A second bioassay with the bacterial pathogen *Pseudomonas solanacearum* also provided evidence that β-HTH can confer effective, protection to transgenic tobacco, relative to control plants. Transgenic tobacco plants were less susceptible to bacterial infection if they contained β-HTH. Tobacco plants which contained both Na-PI and β-HTH were also more resistant to *P. solanacearum,* although in this case, the effect of the two genes was not additive. The severity of disease symptoms was reduced on transgenic plants, compared to untransformed controls.

Chapter Five

Concluding Remarks



"Introducing the *rambo* gene for total resistance may have been a mistake..."

"No, you fool! I said small leaves and big fruit!"

Chapter Five

There is an active debate about whether current use of pesticides and fungicides to control pests and diseases is neither adequate nor sustainable. Furthermore, there is concern, both in developed and developing countries about the effect of chemical pesticides on the environment as well as the development of resistance to the chemicals in pests and pathogens. Biotechnology, together with conventional breeding programs, could make a significant contribution to sustainable agriculture, by improving the tolerance of crop plants to insects and pathogens. However, the problem requires a research strategy which is multi-disciplinary in its approach. A successful outcome depends on the research efforts from plant breeders, pathologists, chemists, agronomists and farmers.

Once the goal of producing disease-resistant and insect-tolerant plants has been set, there are a number of major and minor progressive milestones to be achieved, in order to attain that goal. The approach I have taken, is to explore the use of genetic engineering to transfer potentially useful defence genes into plants and assess those plants for improved resistance to important pests and pathogens. This chapter presents a summary of how each milestone was achieved, along with its significance and implications in terms of past and future research.

5.1 Candidate defence genes

The first requirement in producing genetically modified plants with inherent disease and pest resistance was the choice and availability of genes which encoded defence proteins. The precursor for a proteinase inhibitor from *Nicotiana alata* was unique, in that cleavage of the precursor yields one chymotrypsin inhibitor and four trypsin inhibitors, whereas the majority of other plant PIs have only one or two inhibitory domains. The gene for Na-PI was made available from the University of Melbourne. A second cDNA gene encoding a the precursor of β-hordothionin from barley is also known to be a

potential defence gene. The active and mature β-HTH is released by co- and post-translational cleavage of signal and acidic peptides, respectively in the endosperm of barley. Thus, Na-PI and β-HTH were ideal candidates for transferring into plants because of their potential as defence molecules. However, before this aim could be achieved, it was necessary to show that the precursors could be processed in transgenic plants. Furthermore, as there is increasing evidence that a single defence gene alone may not be adequate to confer durable resistance to plants, it was logical to combine Na-PI and β-HTH, in order to investigate the possibility of pyramiding additive protection by two different mechanisms.

5.2 Plant transformation

5.2.1 Characterising transgenic plants

Having chosen the defence genes, the second prerequisite in obtaining disease-free and insect resistant plants is to be able to transfer the chimeric genes to plant cells and to regenerate whole plants. Tobacco is often used as a model system, primarily to characterise gene expression. In this project transformation of tobacco was achieved with Na-PI and β-HTH both separately and together. Western analysis of transgenic tobacco provided rapid confirmation that both defence molecules were cleaved. Later this observation was extended for Na-PI to include important crop and forage plants. The transfer of Na-PI into pea and subclover was considered important for the following reasons. Firstly, while these legumes had previously been engineered with genes for a bean α-amylase inhibitor or sunflower seed albumin (SSA), respectively (Khan *et al.,* 1994; 1996; Schroeder *et al.,* 1994), this study further confirms the amenability of these legumes to express genes and process the protein products from other unrelated plant families. Second, it is important to demonstrate the effectiveness of defence genes in crop and pasture species. In addition to the PIs that have been transferred into cotton (Thomas *et al.,* 1995b) and rice (Duan *et al.,* 1996; Xu *et al.,* 1996), the transfer of Na-PI into pea and subclover, has provided genetically engineered legumes that have potential for improving plant productivity and are of considerable agricultural importance

to Australia. Finally, as Na-PI has proven effective in tobacco and peas, the gene encoding Na-PI is being considered for use in several other agriculturally important plants, including sugar cane, white clover and apple.

The finding that both the Na-PI and β-HTH precursors had been cleaved to polypeptides of the predicted size, M_r ~6000 and M_r ~8500, respectively, was an important first step in order to maximise the biological activity of the proteins.

5.2.2 Strategies to increase the level of Na-PI and β-HTH in transgenic plants

Transgenic tobacco accumulated Na-PI up to ~0.4% of total soluble leaf protein in T_2 plants and up to 0.5% in F_3 plants, which contained both Na-PI and β-HTH. In pea and subclover, the level of Na-PI was about 0.1% of the soluble leaf protein, indicating a need to increase expression of Na-PI in these legume species. Similarly, although β-HTH was expressed and cleaved in the leaves of transgenic tobacco, the expression of β-HTH needs to be optimised for maximum expression.

In the transformation of tobacco, pea and subclover, the aim was to accumulate Na-PI and β-HTH maximally in leaves. In retrospect, it appears that neither the ASSU nor the CaMV 35S promoters were the most suitable to achieve this. Alternatively its cellular location did not allow for maximum accumulation. Further work is needed to confirm that Na-PI in transgenic plants was located in the vacuoles and to identify the location of β-HTH in transgenic plants. Assuming these molecules were targeted to the vacuole, a more favourable situation would be to express and retain Na-PI and β-HTH elsewhere, preferably where they are more stable than the other leaf proteins. One possible way to increase the level of Na-PI and β-HTH is to divert them away from the vacuole and retain them in the ER. This would require the addition of a C-terminal retention signal, such as the tetrapeptide, KDEL. This strategy has been successfully used to increase the level of this sunflower seed albumin in the leaves of transgenic alfalfa (Tabe *et al.*, 1995) and subclover (Khan *et al.*, 1996). However, the success of this

strategy would depend on the correct co- and post-translational cleavage of the Na-PI- or β-HTH-KDEL modified precursors, and would require specific endoproteinase activity in the ER. One major limitation is that very little is known about endoproteinases in the ER.

Instead of replacing the promoter driving the gene of interest or modifying Na-PI or β-HTH with KDEL, a novel way to increase the level of Na-PI or β-HTH in transgenic plants, would be to substitute the selectable marker with an alternative. For example, in preliminary work with Na-PI, the binary vector was modified by replacing *bar* with *nptII*, which was driven by a strong, constitutive promoter from subclover stunt virus (D. Llewellyn and J. Thistleton; pers. comm.). In tissue culture grown transgenic tobacco plants, Na-PI accumulated maximally to 5% of the soluble protein (J. Thistleton and J. Charity; unpublished data), compared to 1.2% in tissue culture tobacco transformed using *bar* as the selectable marker. However, for plants grown in the glasshouse, Na-PI was not detected at all, even in the leaves of the same plants (J. Charity; unpublished data). It appeared, therefore, that the expression of Na-PI was switched off in glasshouse grown plants, for unknown reasons. This strategy, although successful in elevating temporal expression of Na-PI in tissue culture plants, would not provide durable or reliable tolerance to insects as the level of Na-PI in glasshouse grown plants was inadequate.

5.3 Activity of Na-PI and β-HTH as plant defence proteins

The next step was to demonstrate that the transgenic plants contained antimicrobial or insecticidal activity that could be correlated with the activity of the transgenes. Strong evidence has been presented that Na-PI and β-HTH were biologically active, in that transgenic plants were consistently shown to be more resistant to insects, fungi and bacteria.

5.3.1 Insect resistance

Over half of the insecticides used worldwide are in the cotton and horticultural industries, for the control of *Helicoverpa, Plutella* and *Spodoptera* species (Boulter, 1993). Both *H. armigera* and *H. punctigera* are serious pests of Australian crops, with cotton, grain legumes and tomatoes being the most susceptible (Fitt, 1989; 1991, Zalucki *et al.,* 1991). Therefore, the finding that tobacco and pea transformed with Na-PI were more protected from these two major insect pests may very well find application in producing other resistant transgenic crops - in particular those most affected by *Helicoverpa* species. Moreover, Na-PI was shown to increase larval mortality in a dose-dependent fashion, so it is likely that higher levels of Na-PI in transgenic plants could offer even greater protection from *H. armigera* and *H. punctigera.*

Although thorough experiments demonstrated that detached leaves of transgenic tobacco and pea were more tolerant to insect feeding than untransformed control plants, further confirmatory testing is still required. For instance, it remains to be seen if whole plants containing Na-PI are also more pest-resistant. Furthermore, the experiments presented here and others (e.g. Broadway and Duffy, 1986a; Johnston *et al.,* 1989) observed the effects of PIs on larval mortality and development only until larval pupation. Future insect bioassays should also examine other parameters such as pupal emergence, fecundity and viability of eggs. It would also be important to elucidate the mechanism by which Na-PI slows the growth of *Helicoverpa* species. This might be achieved by determining if Na-PI inhibits trypsin and/or chymotrypsin proteases, in the midgut of *Helicoverpa* species. Such information proved to be important in explaining why transgenic tobacco containing giant taro trypsin inhibitor (GTTI) only marginally affected growth of *H. armigera.* For example, GTTI was shown to decrease the amount of trypsin activity, but larvae were able to compensate for the loss of trypsin proteases by increasing the relative amounts of chymotrypsin and elastase (Y. Wu; pers. comm.).

Resistance of transgenic plants containing Na-PI might potentially extend to include other insect or arthropod pests besides those tested in this study. For example, the gut

proteases of *Agrotis infusa* (common cutworm/Bogong moth; Lepidoptera) and *Teleogryllus commodus* (black field cricket; Orthoptera) were shown to be inhibited by Na-PI, *in vitro* (Heath, 1994), so these are possible test organisms. However, if research in this direction is pursued, it must be with the realisation that the effectiveness of PIs in transgenic plants is very much dependent on the sensitivity of gut proteases in the target organism. This means that while serine proteinases are effective inhibitors of Lepidopteran insects, they are unlikely to be effective against those pests containing cysteine or aspartate gut proteases.

Bioassays aimed at testing transgenic subclover for increased resistance to redlegged earth mite and lucerne flea are still in progress (J. Ridsdill-Smith; G. McDonald pers. comm.).

There is much speculation that exposure of insects to a single toxin could invoke resistance. For this reason, current and future research is aimed at determining if *Helicoverpa* species could be come resistant to Na-PI. And, if resistance develops, it would be important to investigate the mechanism for the evolution of resistance (M. Anderson; pers. comm.). *H. punctigera* could, for example, produce additional proteases to compensate for inhibition by Na-PI and if this was the case, those proteases would need to be identified.

The evidence that insect resistance is delayed when an insect is exposed to two or more toxins simultaneously (Gould, 1994: 1996; Van Rie, 1991; McGaughey and Whalon, 1992; Roush, 1994) has led to a search for genes which interact either additively, or preferably in a synergistic manner. I have shown here, for example, that Na-PI and β-HTH are generally additive in their protective effect, although out of the two molecules, Na-PI is clearly more insecticidal than β-HTH. Another strategy to produce plants containing more than one defence molecule hinges on the multi-domain nature of Na-PI. One or more of the five inhibitory domains of Na-PI could be modified to enhance inhibition of a specific protease, that is, other than trypsin or chymotrypsin. Small modifications at the reactive site at the amino acid level could change the specificity of

the PI (Rolka *et al.,* 1989; Rozycki *et al.,* 1994). For example, a trypsin inhibitor of Na-PI was converted to an elastase inhibitor, simply by substituting arginine at the reactive site with valine (K. Adams; M. Anderson; pers. comm.). This raises the possibility that Na-PI could be modified so that upon cleavage of the precursor, five peptides each with a different specificity are released.

5.3.2 Fungal resistance

Experiments described in this thesis also demonstrated that Na-PI and β-HTH have potential to increase resistance to *Botrytis cinerea*, both in detached leaves and *in planta*. It might be possible to transfer these genes, preferably in combination, into important horticultural crops that are susceptible to *B. cinerea.* As there are over 200 host species affected by *B. cinerea* (Jarvis, 1980), plants chosen for investigation would be restricted to those for which a transformation system exists. For this reason, strawberries, grapes and apples might benefit from the integration of Na-PI, as transformation systems are already available for all three species. Another way to test for biological activity of Na-PI or β-HTH in the leaves of transgenic plants would employ an *in vitro* bioassay where the effect of leaf extracts on growth of a test fungus (e.g. *B. cinerea*) are observed.

5.3.3 Bacterial resistance

Experiments using a bioassay with *P. solanacearum,* although by no means extensive, showed that β-HTH was responsible for increasing disease resistance in transgenic tobacco seedlings. This indicated that β-HTH should be considered amongst the suite of defence genes available for transfer into crop plants to improve productivity. Interestingly, plants which contained β-HTH in combination with Na-PI were not more protected from *P. solanacearum* which suggested that Na-PI was ineffective in inhibiting bacterial growth. This is in agreement with previous *in vitro* bioassays in which purified Na-PI only marginally inhibited the growth of *P. solanacearum* (Dunse, 1992).

5.4 Concluding remarks

In conclusion, it appeared that Na-PI and β-HTH acted differentially, depending upon the target organism. For example, although the effects of the two genes were additive in

providing protection from insect pests, Na-PI was clearly the most insecticidal of the two genes. Moreover, in their provision of increased tolerance to fungal pathogens, although in combination, these genes were additive, the major component of fungal resistance was attributed to the presence of β-HTH. Conversely, in bacterial bioassays transgenic tobacco containing β-HTH were more resistant to bacterial infection, but the addition of Na-PI did not offer further tolerance. The major findings in the insect, fungal and bacterial bioassays are summarised in Table 5-1.

Table 5-1. Differential inhibitory effects of Na-PI and β-HTH on insects, fungi and bacteria

Organism	Transgenic Plant	Gene	Result	Reference (this thesis)
Helicoverpa armigera	Tobacco	Na-PI	• average of 2.3-fold increase in mortality • larvae significantly smaller in first 12 days • up 3 day developmental delay in larval development • decrease in pupation rate	Fig. 3-2
	Peas		• ~1.8-fold increase in mortality • about a 2 day developmental delay in larval development • small decrease in pupation rate	
Helicoverpa punctigera	Tobacco	Na-PI	• ~3.3-fold increase in mortality • up to 7 day developmental delay in larval development	Fig. 3-4
	Peas		• Average of 2.6-fold increase in mortality • About 3 days developmental delay in larval development	
H. armigera	Tobacco	Na-PI	• 1.8-fold increase in mortality • 2 day developmental delay	Fig. 3-5
		β-HTH	• 1.4-fold increase in mortality • larval peak weight was significantly reduced	
		Na-PI and β-HTH	• 3.1-fold increase in mortality • 4 day delay in development • larval peak weight was significantly reduced	

Redlegged earth mite	Subclover	Na-PI	• possible interaction of Na-PI with juvenile RLEM • investigation still in progress	not shown
Botrytis cinerea	Tobacco (T_2 plants)	Na-PI	• Lesions ~16% smaller on transgenic leaves (significant)	Fig. 4-8
		β-HTH	• Very significant reduction in lesion area	
		Na-PI and β-HTH	• Effect of two genes additive, β-HTH contributes major effect, while effect of Na-PI is minor	
Pseudomonas solanacearum	Tobacco	β-HTH	• Seedlings significantly more protected from infection	Fig. 4-10
		Na-PI and β-HTH	• As above, but effect of two genes is not additive	

Abe, Y., Shirane, K., Yokosawa, H., Matsushita, H., Mitta, M., Kato, I. & Ishii, S.I. (1993). Asparaginyl endopeptidase of Jack bean seeds. Purification, characterisation and utility in protein sequence analysis. *J. Biol. Chem.* **268,** 3525-3529.

Abrahams, L.D. & Breuil, C. (1996). Isolation and characterisation of a subtilisin-like serine proteinase secreted by the sap-staining fungus *Ophiostoma piceae. Enzyme Microbiol. Technology* **18,** 133-140.

Agrios, G.N. (1988). Plant Patholgy. (3rd ed). Academic Press, London. pp 1-15.

Alexander, D., Goodman, R.M., Rella, M.G., Glascock, C., Wegmann, K., Friedrich, L., Maddox, D., Ahlgoy, P., Luntz, T., Ward, E. & Ryals, J. (1993). Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis related protein 1a. *Proc. Natl. Acad. Sci. USA* **9,** 7327-7331.

Allefs, S.J.H.M., De Jong, E.R., Florack, E.A., Hoogendoorn, C. & Stiekema, W.J. (1996). *Erwinia* soft rot resistance of potato cultivars expressing antimicrobial peptide tachylplesin I. *Molec. Breeding* **2,** 97-105.

Angerhofer, C.K., Shier, W.T. & Vernon, L.P. (1990). Phospholipase activation in the cytotoxic mechanism of thionin purified from nuts of *Pyrularia pubera. Toxicon.* **28,** 547-554.

Anzai, H., Yoneyama, K. & Yamaguchi, I. (1989). Transgenic tobacco resistant to a bacterial disease by the detoxification of a pathogenic toxin. *Mol. Gen. Genet.* **219,** 492-494.

Atkinson, A. (1992). Expression of a proteinase inhibitor in the pistil of an ornamental tobacco, *Nicotiana alata.* Ph.D. Thesis, School of Botany, University of Melbourne.

Atkinson, A.H., Heath, R.L., Simpson, R.J., Clarke, A.E. & Anderson, M.A. (1993a). Proteinase inhibitors in *Nicotiana alata* stigmas are derived from a precursor protein which is processed into five homologous inhibitors. *Plant Cell* **5,** 203-213.

Atkinson, A.H., Lind, J.L., Clarke, A.E., Anderson, M.A. (1993b). Molecular and structural features of the pistil of *Nicotiana alata. Biochem. Soc. Symp.* **60**, 15-26.

Balandin, T., van der Does, C., Albert, J.M.B., Bol, J.F. & Linthorst, H.J.M. (1995). Structure and induction pattern of a novel proteinase inhibitor class II gene of tobacco. *Plant Mol. Biol.* **27,** 1197-1204.

Balls, A.K. & Hale, W.S. (1940). A suphur-bearing constituent of the petroleum ether extract of wheat flour (preliminary report). *Cereal Chem.* **17,** 243-245.

Balls, A.K., Hale, W.S. & Harris, T.H. (1942). A crystalline protein obtained from a lipoprotein of wheat flour. *Cereal Chem.* **19,** 279-288.

Bekkaoui, F., Datla, R.S.S., Pilon, M., Tautorus, T.E., Crosby, W. & Dunstan, D.I. (1990). The effects of promoters on transient expression in conifer cell lines. *Theor. Appl. Genet.* **79,** 353-359.

Bian, X., Shaw, B.D., Han, Y. & Christeller, J.T. (1996). Midgut proteinase activities in larvae of *Anoplophora glabripennis* (Coleoptera: Cerambycidae) and their interaction with proteinase inhibitors. *Arch. Insect Biochem. Physiol.* **31,** 23-37.

Bloch, C. & Richardson, M. (1991). A new family of small (5 kDa) proteinase inhibitors of insect α-amylase from seeds of sorghum (*Sorghum bicolor* (L) Moench) have sequence homologies with wheat γ-purothionins. *FEBS Lett.* **279,** 101-104.

Bode, W. & Huber, R. (1992). Natural protein proteinase inhibitors and their interaction with proteinases. *Eur. J. Biochem.* **204,** 433-451.

Bohlmann, H. & Apel, K. (1987). Isolation and characterization of cDNAs coding for leaf-specific thionins closely related to the endosperm-specific hordothionin of barley (*Hordeum vulgare* L.). *Mol. Gen. Genet.* **207,** 446-454.

Bohlmann, H. & Apel, K. (1991). Thionins. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **42,** 227-240.

Bohlmann, H. (1994). The role of thionins in plant protection. *Crit. Rev. Plant Sci.* **13,** 1-16.

Bohlmann, H., Clausen, S., Behnke, S., Giese, H., Hiller, C., Reimann-Philipp, U., Schrader, G., Barkholt, V. & Apel, K. (1988). Leaf-specific thionins of barley - a novel class of cell wall proteins toxic to plant-pathogenic fungi and possibly involved in the defence mechanism of plants. *EMBO J.* **7,** 1559-1565.

Boller, T. & Weimken, A. (1987). Dynamics of lysosomal functions in plant vacuoles. In: Plant vacuoles: Their importance in solute compartmentation in cells and their application in plant biotechnology. (Ed.). B. Martin. Plenum Press, New York. pp 134-187.

Bono, F., Savi, P., Tuong, A., Maftouh, M., Pereillo, J.M., Capdevielle, J., Guillemot, J.C., Maffrand, J.P., Herbert, J.M. (1996). Purification and characterisation of a novel protease from culture filtrates of a *Streptomyces* sp. *FEMS Microbiol. Lett.* **141,** 213-220.

Bossi, R., Cole, L., Spier, A.D. & Dewey, F.M. (1994). Monoclonal antibody-based ELISA for detection of mycelial antigens of *Botrytis cinerea* in fruits and vegetables. In: Modern assays for plant pathogenic fungi: identification, detection and quantification. (Eds.) A. Schots, F.M. Dewey, R.P. Oliver. Oxford, England, CAB International. pp 165-172.

Boulter, D. (1993). Insect control by copying nature using genetically engineered crops. *Phytochem.* **34,** 1453-1466.

Boulter, D., Edwards, G.A., Gatehouse, A.M.R., Gatehouse, J.A. & Hilder, V.A. (1990). Additive protective effects of different plant-derived insect resistance genes in transgenic tobacco plants. *Crop Protect.* **9,** 351-354.

Boulter, D.J. & Jongsma, M.A. (1995). Colorado potato beetles (*Leptinotarsa decemlineata*) adapt to proteinase inhibitors induced in potato leaves by methyl jasmonate. *J. Insect Physiol.* **41,** 1071-1078.

Bowles, D.J. (1990). Defence-related proteins in higher plants. *Ann. Rev. Biochem.* **59,** 873-907.

Bowman, H.G. & Hultmark, D. (1987). Cell-free immunity in insects. *Ann. Rev. Microbiol.* **41,** 103-126.

Bradford, M. (1976). A rapid method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* **72,** 248-254.

Brandstadter, J., Rossbach, C. & Theres, K. (1996). Expression of genes for a defensin and a proteinase inhibitor in specific areas of the shoot apex and the developing flower in tomato. *Mol. Gen. Genet.* **252,** 146-154.

Bristow, P.R., McNicol, R.J. & Williamson, B. (1986). Infection of strawberry flowers by *Botrytis cinerea* and its relevance to grey mould development. *Ann. Appl. Biol.* **109,** 545-554.

Broadway, R.M. & Duffey, S.S. (1986a). The effect of dietary protein on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua. J. Insect Physiol.* **32,** 673-680.

Broadway, R.M. & Duffey, S.S. (1986b). Plant proteinase inhibitors: mechansim of action and effect on the growth and digestive physiology of *Heliothis zea* and *Spodoptera exigua. J. Insect Physiol.* **32,** 827-833.

Broadway, R.M. & Villani, M.G. (1995). Does host range influence susceptibility of herbivorous insects to non-host plant proteinase inhibitors? *Entomol. Exp. Appl.* **76,** 303-312.

Broadway, R.M. (1995). Are insects resistant to plant proteinase inhibitors? *J. Insect Physiol.* **41,** 107-116.

Broadway, R.M. (1996). Dietary proteinase inhibitors alter complement of midgut proteases. *Arch. Insect Biochem. Physiol.* **32,** 39-53.

Broekaert, W.F., Marien, W., Terras, F.R.G., Proost, P., Van Damme, J., Dillen, L., Claeys, M., Reese, S.B., Vanderleyden, J. & Cammue, B.P.A. (1992). Antimicrobial peptides from *Amaranthus caudatus* seeds with sequence homology to the cysteine/glycine-rich domain of chitin binding proteins. *Biochem.* **31,** 4308-4315.

Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A. & Osborn, R.W. (1995). Plant defensins: Novel antimicrobial peptides as components of the host defence system. *Plant Physiol.* **108,** 1353-1358.

Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C.J & Broglie, R. (1991). Transgenic plants with enhanced resistance to the fungal pathogen *Rhizocotonia solani. Science* **254,** 1194-1197.

Brown, A.E. & Adikaram, N.K.B. (1983). A role for pectinase and protease inhibitors in fungal rot development in tomato fruits. *Phytopath. Z.* **106,** 239-251.

Bruix, M., Gonzalez, C., Santoro, J. Soriano, F., Rocher, A. & Mendez, E. (1995). H-1-NMR studies on the structure of a new thionin from barley endosperm. *Biopolymers* **36,** 751-753.

Bryant, J., Green, T.R., Gurusaddaiah, T. & Ryan, C.A. (1976). Proteinase inhibitor II from potatoes: Isolation and characterisation of its protomer components. *Biochem.* **15,** 3418-3423.

Brzin. J. & Kidric., M. (1995). Proteinases and their inhibitors in plants: Role in normal growth and in response to various stress conditions. In: Biotechnology and Genetic Engineering Review. (Ed.). M.P. Tombs. A. Intercept, Andover. pp421-467.

Buddenhagen, I.W. & Kelman, A. (1964). Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum. Ann. Rev. Phytopathol.* **2,** 203-230.

Burgess, E.P.J., Stevens, P.S., Keen, G.K., Laing, W.A. & Christeller, J.T. (1991). Effects of protease inhibitors and dietary protein level on the black field cricket *Teleogryllus commodus. Entomol. Exp. Appl.* **61,** 123-130.

Cammue, B.P.A., De Bolle, M.F.C., Schoofs, H.M.E., Terras, F.R.G., Thevissen, K., Osborn, R.W., Rees, S.B. & Broekaert, W.F. (1994). Gene-encoded antimicrobial peptides from plants. In: Antimicrobial peptides. (*Ciba Foundation Symposium 186*). Wiley, Chichester. pp 91-106.

Cammue, B.P.A., De Bolle, M.F.C., Terras, F.R.G., Proost, P., Van Damme, J., Rees, S.B., Vanderleyden, J. & Broekaert, W.F. (1992). Isolation and characterisation of a novel class of plant antimicrobial peptides of *Mirabilis jalapa* L. seeds. *J. Biol. Chem.* **267,** 2228-2233.

Carmona, M.J., Hernández-Lucas, San Martin, C, González, P. & García-Olmedo, F. (1993a). Subcellular localization of type I thionins in the endosperms of wheat and barley. *Protoplasma.* **173,** 1-7.

Carmona, M.J., Molina, A., Fernandez, J.A., Lopez-Fando, J.J. & García-Olmedo, F. (1993b). Expression of the α-thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens. *Plant J.* **3,** 457-462.

Carr, J.P. & Klessig, D.F. (1989). The pathogenesis related proteins of plants. In: Genetic Engineering: Principles and Methods, Vol 11. (Ed.). Setlow, J.K Plenum Press, New York. pp 65-109.

Carrasco, L., Vázquez, Hernández-Lucas, C., Carbonero, P. & García-Olmedo, F. (1981). Thionins: Plant peptides that modify membrane permeability in cultured mammalian cells. *Eur. J. Biochem.* **116,** 185-189.

Castagnaro, A, Maraña, C, Carbonero, P, García-Olmedo, F. (1992). Extreme divergence of a novel wheat thionin generated by a mutational burst specifically affecting the mature protein domain of the precursor. *J. Mol. Biol.* **224,** 1003-1009.

Castagnaro, A, Maraña, C, Carbonero, P, García-Olmedo, F. (1994). cDNA cloning and nucleotide sequences of α_1 and α_2 thionins from hexaploid wheat endosperm. *Plant Physiol.* **106,** 1221-1222.

Chandler, P.M., Higgins, T.J.V., Randall, P.J. & Spencer, D. (1983). Regulation of legumin levels in developing pea seeds under conditions of sulfur deficiency: Rates of legumin synthesis and levels of legumin mRNA. *Plant Physiol.* **71**, 47-54.

Charity, J.A., Bittisnich, D., Anderson, M.A. & Higgins, T.J.V. (1997). Transgenic tobacco and peas containing a proteinase inhibitor from *Nicotiana alata* have increased insect resistance. *Molec. Breeding.* (in preparation).

Christeller, J.T. & Shaw, B.D. (1989). The interactions of a range of serine proteinase inhibitors with bovine typsin and *Costelytra zealandica* trypsin. *Insect Biochem.* **19,** 233-241.

Christeller, J.T., Laing, W.A., Markwick, N.P. & Burgess, E.P.J. (1992). Midgut protease activities in 12 phytophagous Lepidopteran larvae: Dietry and protease inhibitor interactions. *Insect Biochem. Molec. Biol.* **22,** 735-746.

Coley-Smith, J.R., Verhoeff, K. & Javis, W.R. (1980). The biology of *Botrytis.* Academic Press, New York, pp 311-330.

Colilla, F.J., Rocher, A. & Mendez, E. (1990). γ-Purothionins: amino acid sequence of two polypeptides of a new family of thionins from wheat endosperm. *FEBS Lett.* **270,** 191-194.

Constabel, C.P. & Brisson, N. (1995). Stigma and vascular-specific expression of the PR-10a genes of potato: A novel pattern of expression of a pathogenesis related gene. *Molec. Plant-Microbe Interact.* **8,** 104-113.

Cooper, J.B., Chen, J.A., van Holst G.J., Varner, J.E. (1987). Hydroxyproline-rich glycoproteins of plant cell walls. *Trends Biochem. Sci.* **12,** 24-27.

Cornelissen, B.J.C. & Melchers, L.S. (1993). Strategies for control of fungal diseases with transgenic plants. *Plant Physiol.* **101,** 709-712.

Craig, S. & Beaton, C.D. (1996). A simple cryo-SEM method for delicate plant tissues. *J. Microscopy* **182,** 102-105.

Cutt, J.R., Harpster, M.H., Dixon, D.C., Carr, J.P., Dunsmuir, P. & Klessig, D.F. (1989). Disease response to tobacco mosaic virus in transgenic tobacco plants that constitutively express the pathogenesis-related PR-1b gene. *Virology* **173,** 89-97.

Czapla, T.H. & Lang, B.A. (1990). Effect of plant lectins on the development of European cornborer (Lepidoptera: Pyralidae) and Southern corn root-worm (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* **83,** 2480-2485.

Daly, J.C. (1993). Ecology and genetics of insecticide resistance in *Heliocoverpa armigera*: Interactions between selection and gene flow. *Genetica* **90**, 217-226.

De Almeida, E.R.P., Gossele, V., Muller, C.G., Dockx, J., Reynaerts, A., Botterman, J., Krebbers, E. & Timko, M.P. (1989). Transgenic expression of two marker genes under the control of an *Arabidopsis rbsS* promoter: Sequences encoding the Rubisco transit peptide increase expression levels. *Mol. Gen. Genet.* **218,** 78-86.

De Bolle, M.F.C., Osborn, R.W., Goderis, I.J., Noe, L., Acland, D., Hart, C.A., Torrekens, S., Van Leuven, F. & Broekaert, W.F. (1996). Antimicrobial peptides from *Mirabilis jalapa* and *Amaranthus caudatus:* Expression, processing, localization and biological activity in transgenic tobacco. *Plant Mol. Biol.* **31,** 993-1008.

Demina, N.S. & Lysenko, S.V. (1996). Collagenolytic enzymes from microorganisms. *Microbiol.* **65,** 257-265.

Dempsey, S.A. & Klessig, D.F. (1994). Salicylic acid, active oxygen species and systemic acquired resistance in plants. *Trends Cell Biol.* **4,** 334-338.

Ditta, G., Stanfield, S., Corbin, D. & Helsinki, D.R. (1980). Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti. Proc. Natl. Acad. Sci. USA* **77,** 7347-7351.

Dowson, W.J. (1957). Plant diseases due to bacteria. Cambridge, University Press. pp 127-129.

Duan, X., Li, X., Xue, Q., Abo-El-Saad, M., Xu, D. & Wu, R. (1996). Transgenic rice plants harbouring an introduced potato proteinase inhibitor II gene are insect resistant. *Nature Biotech.* **14,** 494-498.

Dunaevskii, Y., Pavlikova, E.B., Belyakova, G.A. & Belozerskii, M.A. (1995). Physiological role of protease inhibitors in plants - two groups of active inhibitors in buckwheat seed. *Molec. Biol.* **29,** 747-750.

Dunse, K. (1992). Evidence for the resistance of *Nicotiana alata* pistils to pathogen invasion. Honours thesis. School of Botany, University of Melbourne, Australia.

Düring, K., Porsch, P., Fladung, M. & Lörz, H. (1993). Transgenic potato plants resistant to the phytopathogenic bacteria *Erwina carotovora. Plant J.* **3,** 587-598.

Edmonds, H.S., Gatehouse, L.N., Hilder, V.A. & Gatehouse, J.A. (1996). The inhibitory effects of the cysteine protease inhibitor, oryzacystatin, on digestive proteases and larval survival and development of the southern corn rootworm (*Diabrotica undecimpunctata howardi*). *Entomol. Exp. Appl.* **78,** 83-94.

Elden, T.C. (1995). Selected proteinase inhibitor effects on alfafa weevil (Coleoptera: Curculionidae) growth and development. *J. Econ. Entomol.* **88,** 1586-1590.

Enyedi, A.J., Yalpani, N., Silverman, P. & Raskin, I. (1992). Signal molecules in systemic plant resistance to pathogens and pests. *Cell* **70,** 879-886.

Epple, P., Apel, K. & Bohlmann, H. (1995). An *Arabidopsis thaliana* gene is inducible via a signal transduction pathdway different from that for pathogenesis-related proteins. *Plant Physiol.* **109,** 813-820.

Etzler, M.A. (1985). Plant lectins: molecular and biological aspects. *Ann. Rev. Plant Physiol.* **36**, 209-234.

Evans, J., Wang, Y., Shaw, K.P. & Vernon, L.P. (1989). Cellular responses to *Pyrularia* thionin are mediated by Ca^{2+} influx and phospholipase A2 activation and are inhibited by thionin tyrosine iodination. *Proc. Natl. Acad. Sci. USA.* **86,** 5849-5853.

Farmer, E.E. & Ryan, C.A. (1992). Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. *Plant Cell* **4,** 129-134.

Farmer, E.E., Johnson, R.R. & Ryan, C.A. (1992). Regulation of expression of proteinase inhibitor genes by methyl jasmonic acid. *Plant Physiol.* **98,** 995-1002.

Fernandez de Caleya, R., Gonzalez-Pascual, B., García-Olmedo, F. & Carbonero, P. (1972). Susceptibility of phytopathogenic bateria to wheat purothionins *in vitro. Appl. Microbiol.* **23,** 998-1000.

Fischhoff, D.A., Bowdish, K.S., Perlak, F.J., Marrone, P.G., McCormick, S.H., Niedermeyer, J.G., Dean, D.A., Kusano-Kretzmer, K., Mayer, E.J., Rochester, D.E., Rogers, S.G. & Fraley, R.T. (1987). Insect tolerant tomato plants. *Bio/Technology* **5,** 807-813.

Fisher, R., Behnke, S. & Apel, K. (1989). The effect of chemical stress on the intercellular fluid of barley leaves. *Planta* **178,** 61-68.

Fitt, G.P. (1989). The ecology of *Heliothis* species in relation to agroecosystems. *Ann. Rev. Entomol.* **34,** 17-52.

Fitt, G.P. (1991). Host selection in the *Heliothinae.* In: Reproductive behaviour in insects: Individuals and populations. (Eds.). W. Bailey & J. Ridsdill-Smith, Chapman and Hall, London, pp 173-201.

Florack, D.E.A. & Stiekema W.J. (1994). Thionins: properties, possible biological roles and mechanisms of action. *Plant Mol. Biol.* **26,** 25-37.

Florack, D.E.A., Dirkse, W.G., Visser, B., Heidekamp, F. & Stiekema, W.J. (1994). Expression of biologically active hordothionins in tobacco. Effects of pre- and pro-sequences at the amino and carboxyl termini of the hordothionin precursor on mature protein expression and sorting. *Plant Mol. Biol.* **24,** 83-96.

Florack, D.E.A., Visser, B., De Vries, P.M., Van Vuurde, J.W.L. & Stiekema, W.J. (1993). Analysis of the toxicity of purothionins and hordothionins for plant pathogenic bacteria. *Neth. J. Plant Pathol.* **99,** 259-268.

Fujimoto, H., Itoh, K., Yamamoto, M, Kyozuka, J. & Shimamoto, K. (1993). Insect resistant rice generated by introduction of a modified δ-endotoxin gene of *Bacillus thuringiensis. Bio/Technology* **11**, 1151-1155.

García-Olmedo, F., Carbonero, P., Hernández-Lucas, C., Paz-Ares, J., Ponz, F., Vicente, O., and Sierra, J.M. (1983). Inhibition of eukaryotic cell-free protein synthesis by thionins from wheat endosperm. *Biochem. Biophys. Acta.* **740,** 52-56.

García-Olmedo, F., Carmona, M., López-Fando, J.J., Castagnaro, A., Molina, A., Hernández-Lucas, C., Carbonero, P. (1992). Characterisation and analyisis of thionin genes. In: (Eds.). T. Boller, F. Meins. Genes involved in plant defence. Springer, Wien New York, pp 283-302.

García-Olmedo, F., Rodríguez-Palenzuela, P., Hernández-Lucas, C., Ponz, F., Maraña, C., Carmona, M.J., López-Fando, J., Fernandez, J.A., and Carbonero, P. (1989). The Thionins: a protein family that includes purothionins, viscotoxins and crambins. *Oxford Surv. Plant Mol. Cell Biol.* **6**, 31-60.

García-Olmedo, F., Salcedo, G., Sanchez-Monge, R., Gomez, L., Royo, J. & Carbonero, P. (1987). Plant proteinaceous inhibitors of proteinases and α-amylases. *Oxford Surv. Plant Mol. Cell Biol.* **4,** 275-334.

Gatehouse, A.M.R. & Boulter, D. (1983). Assessment of the antimetabolic effects of trypsin inhibitors from cowpea (*Vigna unguiculata*) and other legumes on development of the brucid beetle *Callosobruchus maculatus. J. Sci. Food Agric.* **34,** 345-350.

Gatehouse, A.M.R. & Hilder, V.A. (1994). Genetic Manipulation of crops for insect resistance. In: Molecular Biology in Crop Protection. (Ed.). G. Marshall, D. Waters. Chapman & Hall, London. pp 177-201.

Gatehouse, A.M.R. Dewey, F.M., Dove, J., Fenton, K.A. & Pusztai. (1984). Effect of seed lectins from *Phaseolus vulgaris* on the development of larvae of *Callosobruchus maculatus;* Mechanism of toxicity. *J. Sci. Food Agric.* **35,** 373-380.

Gatehouse, A.M.R., Fenton, K.A., Jepson, I. & Pavey, D.J. (1986). The effects of α-amylase inhibitors on insect storage pests: Inhibition of α-amylase *in vitro* and effects on development *in vivo. J. Sci. Food Agric.* **37,** 727-734.

Gatehouse, A.M.R., Gatehouse, J.A., Dobie, P., Kilminster, A.M. & Boulter, D. (1979). Biochemical basis of insect resistance in *Vigna unguiculata. J. Sci. Food Agric.* **30,** 948-958.

Gatehouse, A.M.R., Powell, K.S., Peumans, W.J., Van Damme, E. & Gatehouse, J.A. (1995). Insecticidal properties of plant lectins: their potential in plant protection. In: Lectins: Biomedical perspective. (Ed.). A.J. Pusztai. Taylor and Francis. pp 35-57.

Gausing, K. (1987). Thionin genes specifically expressed in barley leaves. *Planta.* **171,** 241-246.

Geoffroy, P., Legrand, M. & Fritig, B. (1990). Isolation and characterization of a proteinase inhibitor of microbial proteinases induced during the hypersensitive reaction of tobacco to tobacco mosaic virus. *Mol. Plant Microb. Interact.* **3,** 327-333.

Gerlach, W.L. & Bedbrook, J.R. (1979). Cloning and characterisation of ribosomal RNA genes from wheat and barley. *Nucleic Acid Res.* **7**, 1869-1885.

Gillespie, D.J. (1994). Pasture Legume Recommendations for sowing in 1993 and 1994. *Department of Agriculture W.A Bulletin.* **4249.**

Gincel, E., Simorre, J.P., Caille, A., Marion, D., Ptak, M. & Vovelle, F. (1994). Three-dimensional structure in solution of a wheat lipid-transfer protein from multidimensional ^{1}H-NMR data. A new folding for lipid carriers. *Eur. J. Biochem.* **226,** 413-422.

Goldstein, I.J. & Hayes, C.E. (1978). The lectins: Carbohydrates-binding proteins of plants and animals. *Adv. Carbohyd. Chem. Biochem.* **35**, 127-340.

Gould, F. (1986). Simulation models for predicting durability of insect-resistant germ plasm: a deterministic diploid, two-locus model. *Environ. Entomol.* **15,** 1-10.

Gould, F. (1994). Potential and problems with high-dose strategies for pesticidal engineered crops. *Biocontrol Sci. Technol.* **4,** 451-463.

Gould, F., Martinez-Ramirez, A., Anderson, A., Ferre, J., Silva, F.J. & Moar, W.J. (1992). Broad-spectrum resistance to *Bacillus thuringiensis* toxins against *Heliothis virescens. Proc. Natl. Acad. Sci., USA.* **89**, 7986-7990.

Graham, J., Jones, D.A. & Lloyd, A.B. (1979). Survival of *Pseudomonas solanacearum* race 3 in plant debris and in latently infected potato tubers. *Phytopathol.* **69,** 1100-1103.

Graham, S., Hall, G., Pearce, G. & Ryan, C.A. (1986). Regulation of synthesis of proteinase inhibitors I and II mRNAs in leaves of wounded tomato plants. *Planta.* **169,** 399-405.

Graham, S., Pearce, G., Merryweather, J., Titani, K., Ericsson, L. & Ryan, C.A. (1985a). Wound-induced proteinase inhibitors from tomato leaves. I. The cDNA-deduced primary structure of pre-inhibitor I and its post-translational processing. *J. Biol. Chem.* **260,** 6555-6560.

Graham, S., Pearce, G., Merryweather, J., Titani, K., Ericsson, L. & Ryan, C.A. (1985b). Wound-induced proteinase inhibitors from tomato leaves. II. The cDNA-deduced primary stucture of pre-inhibitor II. *J. Biol. Chem.* **260,** 6561-6564.

Green, T.R. & Ryan, C.A. (1972). Wound-induced proteinase inhibitors in plant leaves: A possible defence mechanism against insects. *Science* **175,** 776-777.

Grinzburg, B.Z. (1961). Evidence for a protein gel structure cross-linked by metal cations in the intercellular cement of plant tissues. *J. Exp. Bot.* **12,** 85-107.

Gutierrez, C., Sanchez-Monge, R., Gomez, L., Ruiz-Tapiador, M., Castanera, P. & Salcedo, G. (1990). α-Amylase activities of agricultural insect pests are specifically affected by different inhibitor preparations from wheat and barley endosperms. *Plant Sci.* **72,** 37-44.

Hain, R., Biesseler, B., Kindl, H., Schröeder, G. & Stöcker, R. (1990). Expression of a stilbene synthase gene in *Nicotiana tobacum* results in synthesis of the phytoalexin resveratrol. *Plant Mol. Biol.* **15,** 325-335.

Hain, R., Reif, H., Krause, E., Langebartels, R., Kindle, H., Vornam, B., Wiesse, W., Schmelzer, E., Schreier, P.H., Stöcker, R.H. & Stenzel, K. (1993). Disease resistance results from foreign phytoalexin expression in a novel plant. *Nature* **361,** 153-156.

Hames, B.D. & Rickwood, D. (1990). Gel electrophoresis of proteins. A practical approach. 2nd Ed. IRL Press, Oxford.

Hara-Nishimura, I., Inoue, K. & Nishimura, M. (1991). A unique vacuolar processing enzyme responsible for conversion of several proprotein precursors into the mature forms. *FEBS Lett.* **294,** 89-93.

Hass, G.M., Hermodson, M.A., Ryan, C.A., Gentry, L. (1982). Primary structures of two low molecular weight proteinase inhibitors from potatoes. *Biochem.* **21,** 752-756.

Heath, R.L. (1994) Characterisation and processing of a multidomain proteinase inhibitor from *Nicotiana alata.* Ph.D Thesis. School of Botany, University of Melbourne.

Heath, R.L., Barton, P.A., Simpson, R.L., Reid, G.A., Lim, G. & Anderson, M.A. (1995). Characterisation of protease processing sites in a multidomain proteinase inhibitor precursor from *Nicotiana alata*. *Eur. J. Biochem.* **230,** 250-257.

Heath, R.L., McDonald, G., Bateman, K., Christeller, J.T., Lee, M., Vanheeswijck, R. & Anderson, M. (1997). The potential of proteinase inhibitors from *Nicotiana alata* for enhancing plant resistance to insect pests. (in preparation).

Hedrick, S.A., Bell, J.N., Belloer, T. & Lamb, C.J. (1988). Chitinase cDNA cloning and mRNA induction by fungal elicitor, wounding and infection. *Plant Physiol.* **86**, 182-186.

Heilbronn, J. & Lyon, G.D. (1990). Ineffectuality of potato protease inhibitor on the extracellular protease from *Erwinia carotovora* subsp. *carotovora. J. Appl. Bacteriol.* **69,** 25-29.

Hernández-Lucas, C., Fernandez de Caleya, R. & Carbonero, P. (1974). Inhibition of brewer's yeasts by wheat purthionins. *Appl. Microbiol.* **28,** 165-168.

Hernández-Lucas, C., Royo, J., Paz-Arez, J., Ponz, F., García-Olmedo, F. & Carbonero, P. (1986). Polyadenylation site heterogeneity in mRNA encoding the precursor of the barley toxin β-hordothionin. *FEBS Lett.* **200,** 103-106.

Higgins, T.J.V. & Spencer, D. (1991). The expression of a chimeric cauliflower mosaic virus (CaMV-35S)-pea vicilin gene in tobacco. *Plant Sci.* **74,** 89-98.

Higgins, T.J.V., Newbigin, E.J., Spencer, D., Llewellyn, D.J. & Craig, S. (1988). The sequence of a pea vicilin gene and its expression in transgenic tobacco plants. *Plant Mol. Biol.* **11,** 683-695.

Hilder, V.A., Gatehouse, A.M.R., Sheerman, S.E., Barker R.F. & Boulter, D. (1987). A novel mechanism of insect resistance engineered into tobacco. *Nature* **300,** 160-163.

Hilder, V.A., Powell, K.S., Gatehouse, A.M.R., Gatehouse, J.A., Gatehouse, L.N., Shi, Y., Hamilton, W.D.O., Merryweather, A., Newell, C.A., Timans, J.C., Peumans, W.S., Van Damme, E. & Boulter, D. (1995). Expression of snowdrop lectin in transgenic tobacco plants results in added protection against aphids. *Transgenic Res.* **4,** 18-26.

Hobbs, S.L.A., Kpodar, P. & Delong, C.M.O. (1990). The effect of T-DNA copy number, position and methylation on reporter gene expression in transgenic tobacco. *Plant Mol. Biol.* **15,** 851-864.

Hofte, H. & Whitely, H.R. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* **53**, 242-255.

Horsh, R., Fry, J., Hoffman, N., Eichholtz, P., Rogers, R. & Fraley, T. (1985). A simple method for transferring genes into plants. *Science* **227,** 1229-1231.

Huesing, J.E., Shade, R.E., Chrispeels, M.J. & Murdock, L.L. (1991). α-Amylase inhibitor, not phytohaemagglutinin, explains resistance of common bean seeds to cowpea weevil. *Plant Physiol.* **96,** 993-996.

Hussain, A., & Kelman, A. (1958). Relation of slime production to the mechansisms of wilting and pathogenicity of *Pseudomonas solanacearum*. *Phytopathol.* **48,** 155-165.

Isaac, S. (1992). Fungal-plant interactions. Chapman & Hall, London.

Jach, G., Gornhardt, B., Mundy, J., Logemann, J., Pinsdorf, P., Leah, R., Schell, M. & Maas, C. (1995). Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. *Plant J.* **8,** 97-109.

Jarvis, W.R. (1980). Taxonomy. In: The biology of *Botrytis*. (Eds.). J.R. Coley-Smith, K. Verhoeff, W.R. Jarvis. Academic Press, London. pp 1-18.

Jaynes, J.M., Xanthopoulos, K.G., Destéfano-Beltrán, L. & Dodds, J.H. (1987). Increasing bacterial disease resistance in plants utilizing antibacterial genes from insects. *Bioessays* **6,** 262-270.

Jenkins, S. & Kelman, A. (1976). Techniques for the study of *Pseudomonas solanacearum*. In: Proceedings of the first international planning conference and workshop on the

ecology and control of bacterial wilt caused by *Pseudomonas solanacearum.* (Eds.). L. Sequeira, A. Kelman. North Carolina. pp 143-147.

Johnson, R., Narvaez, J., An, G. & Ryan, C.A. (1989). Expression of proteinase inhibitors I and II in transgenic tobacco plants: Effects on natural defence against *Manduca sexta* larvae. *Proc Natl. Acad. Sci. USA* **86,** 9871-9875.

Johnson, T.C., Wada, K., Buchanan, B.B. & Holmgren, A. (1987). Reduction of purothionin by the wheat seed thioredoxin system. *Plant Physiol.* **85,** 446-451.

Johnston, K.A., Gatehouse, J.A. & Anstee, J.H. (1993). Effects of soybean proteinase inhibitors on the growth and development of larval *Heliocoverpa armigera. J. Insect Physiol.* **39,** 657-664.

Johnston, K.A., Lee, M.J., Gatehouse, J.A. & Anstee, J.H. (1991). The partial purification and characterisation of serine protease activity in midgut of larval *Helicoverpa armigera. Insect Biochem.* **21,** 389-397.

Johnstone, G.R. & McLean, G.P. (1987). Virus diseases of subterranean clover. *Ann. Appl. Biol.* **110,** 421-440.

Jones, B.L. & Meredith, P. (1982). Inactivation of alpha-amylase activity by purathionin. *Cereal Chem.* **59,** 321.

Jones, B.L., Lookhart, G.L. & Johnson, D.E. (1985). Improved separation and toxicity analysis methods for purathionins. *Cereal Chem.* **62,** 327-331.

Jones, J.D.G., Gilbert, D.E., Grady, K.L. & Jorgensen, R.A. (1987). T-DNA structure and gene expression in petunia plants transformed by *Agrobacterium tumefaciens* C58 derivatives. *Mol. Gen. Genet.* **207,** 478-485.

Jongedijk, E., Tigelaar, H., van Roekel, J.S.C., Bres-Vloemans, S.A., Dekker, I., van den Elzen, P.J.M., Cornelissen, B.J.C. & Melchers, L.S. (1995). Synergistic activity of chitinases and β-1,3-glucanases enhances fungal resistance in transgenic tomato plants. *Euphytica* **85,** 175-180.

Jongsma, M.A., Bakker, P.L., Peters, J., Bosch, D. & Steikema, W.J. (1995). Adapation of *Spodoptera exigua* larvae to plant proteinase inhibitors by induction of gut proteinase activity insensitive to inhibition. *Proc. Natl. Acad. Sci. USA.* **92,** 8041-8045.

Jongsma, M.A., Bakker, P.L., Visser, B. & Stiekema, W.J. (1994). Trypsin inhibitor activity in mature tobacco and tomato plants is mainly induced locally in response to insect attack, wounding and virus infection. *Planta* **195,** 29-35.

Kang, J.W., Mao, G.Z. & He, L.Y. (1994). Factors involved in virulence and pathogenicity of *Pseudomonas solanacearum* and their role in pathogenesis. In: Groundnut bacterial wilt in Asia: Proceedings of the third working group meeting. (Ed.) V.K. Mehan, D. McDonald. ICRISAT, India. pp 53-62.

Karey, K.P. & Sirbasku, D.A. (1989). Glutaraldehyde fixation increases retention of low molecular weight proteins (growth factors) transferred to nylon membranes for western blot analysis. *Analyt. Biochem.* **178,** 255-259.

Keil, M., Sanchez-Serrano, J.J. & Willmitzer, L. (1989). Both wound-inducible and tuber-specific expression are mediated by the promoter of a single member of the potato proteinase inhibitor II gene family. *EMBO J.* **8,** 1323-1330.

Keil, M., Sanchez-Serrano, J.J., Schell, J. & Willmitzer, L. (1990). Localization of elements important for the wound-inducible expression fo a chimeric potato proteinase inhibitor II-CAT gene in transgenic tobacco plants. *Plant Cell* **2,** 61-70.

Khan, M.R.I., Ceriotti, A., Tabe, L., Aryan, A., McNabb, M., Moore, A., Craig, S., Spencer, D. & Higgins, T.J.V. (1996). Accumulation of a sulphur-rich seed albumin from sunflower in the leaves of transgenic subterranean clover (*Trifolium subterraneum* L.) *Transgenic Res.* **5,** 179-185,

Khan, M.R.I., Tabe, L.M., Heath, L.C., Spencer, D. & Higgins, T.J.V. (1994). *Agrobacterium*-mediated transformation of subterranean clover (*Trifolium subterraneum* L.). *Plant Physiol.* **105,** 81-88.

Kiraly, Z. (1980). Defences triggered by the invader: hypersensitivity. In: Plant Defence, Vol V. (Eds.). J.G. Horstfall, E.B. Cowling. Academic Press, New York. pp 201-224.

Klessig, D.F. & Malamy J. (1994). The salicylic acid signal in plants. *Plant Mol. Biol.* **26,** 1439-1458.

Kozak, M. (1989). A scanning model for translation: An update. *J. Cell Biol.* **108,** 229-241.

Kramer, K.J., Klassen, L.w., Jones, B.L., Spiers, R.D. & Kammer, A.E. (1979). Toxicity of purothionin and its homologues to the tobacco hornworm, *Manduca sexta* (L.) (Lepidoptera: Sphingidae). *Toxicol. Appl. Pharmacol.* **48,** 179-183.

Krebbers, E., Herdies, L., De Clercq, A., Seurinck, J., Leemans, J., Damme, J., Segura, M., Gheysen, G., Van Montagu, M., Vankerckhove, J. (1988a). Determination of the processing sites of *Arabidopsis* 2S albumins and characterisation of the complete gene family. *Plant Physiol.* **87,** 859-866.

Krebbers, E., Seurinck, Herdies, L., Cashmore, A.R. & Timko, M.P. (1988b). Four genes in two diverged subfamilies encode the ribulose-1,5-bisphosphate carboxylase small subunit polypeptides of *Arabidopsis thaliana. Plant Mol. Biol.* **11,** 745-759.

Kuc, J. & Williams, E.B. (1962). Production of proteolytic enzymes by four pathogens of apple fruit. *Phytopath.* **52,** 739.

Kumar, P.A. & Sharma, R.P. (1994). Genetic engineering of insect-resistant crop plants with *Bacillus thuringiensis* crystal protein genes. *J. Plant Biochem. Biotech.* **3,** 3-8.

Kuroda, M., Ishimoto, M., Suzuki, K., Kondo, H, Abe, K, Kitamura, K. & Arai, S. (1996). Oryzacystatins exhibit growth-inhibitory and lethal effects on different species of bean insect pests, *Callosobruchus chinensis* (Coleoptera) and *Riptortus clavatus* (Hemiptera). *Biosci. Biotech. Biochem.* **60,** 209-212.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680-685.

Lamb, C.J., Ryals, J., Ward, E.R. & Dixon, R.A. (1992). Emerging strategies for enhancing crop resistance to microbial pathogens. *Bio/Technology* **10,** 1436-1445.

Lamb, J., Poddar A. & Riverton, S.A. (1987). Grain Legume Handbook. *S.A. Peagrowers Co-operative Ltd.*

Lambin, P. (1978). Reliability of molecular weight determination of proteins by polyacrylamide gradient gel electrophoresis in the presence of sodium dodecyl sulphate. *Anal. Biochem.* **85,** 114-125.

Lambkin, I., Hamilton, A.J. & Hay, R.J. (1996). Purification and characterisation of a novel 34 000-M(R) cell-associated proteinase from the dermatophte *Trichophyton rubrum. FEMS Immunol. Medical Microbiol.* **13,** 131-140.

Larocque, A.M. & Houseman, J.G. (1990). Effect of ingested soybean, ovomucoid and corn protease inhibitors on digestive processes of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *J. Insect Physiol.* **36,** 691-697.

Laskowski Jr., M. & Kato, I. (1980). Protein inhibitors of proteases. *Ann. Rev. Biochem.* **49,** 593-626.

Laurière, M. (1993). A semidry electroblotting system efficiently transfers both high- and low-molecular-weight proteins separated by SDS-PAGE. *Analyt. Biochem.* **212,** 206-211.

Lawton, M.A. & Lamb, C.J. (1987). Transcriptional activation of plant defence genes by fungal elicitor, wounding and infection. *Mol. Cell Biol.* **7**, 335-341.

Leah, R., Tommerup, H., Svendsen, I. & Mundy, J. (1991). Biochemical and molecular characterisation of three barley seed proteins with antifungal properties. *J. Biol. Chem.* **266,** 1564-1573.

Leiner, I.E. & Kadade, M.L. (1980). Protease inhibitors. In: *Toxic constituents of plant foodstuffs.* (Ed.). I.E. Leiner. Academic Press, London. pp 7-21.

Leplé, J.C., Bonadé-Bottino, M., Augustin, S., Pilate, G., Dumanois Lê Tân, V., Delplanque, A., Cornu, D. & Jouanin, L. (1995). Toxicity to *Chrysomela tremulae* (Coleoptera: Chrysomelidae) of transgenic poplars exressing a cysteine proteinase inhibitor. *Molec. Breeding* **1,** 319-328.

Lepoivre, P. & Boy, N. (1983). Effect of pea lectin on the infection of pea by *Mycosphaerella pinodes* (Berk. & Blox.) Verstergr. *Parasitica* **39,** 145-149.

Leung, D.W.M. (1991). Involvement of a plant chitinase in sexual reproduction of higher plants. *Phytochem.* **31**, 1899-1900.

Linn, F., Heidmann, I., Saedler, H. & Meyer, P. (1990). Epigenetic changes in the expression of the maize A1 gene in *Petunia hybrida:* Role of numbers of integrated gene copies and state of methylation. *Mol. Gen. Genet.* **222,** 329-336.

Linthorst, H.J.M., Meuwissen R.L.J., Kauffmann, S. & Bol, J.F. (1989). Constitutive expression of pathogenesis-related proteins PR-1 and PR-S in tobacco has no effect on virus infection. *Plant Cell* **1,** 285-291.

Logemann, J., Jach, G., Tommerup, H., Mundy, J. & Schell, J. (1992). Expression of a barley ribosome-inactivating protein leads to increase fungal protection in transgenic tobacco plants. *Bio/Technology* **10,** 305-308.

Lorito, T., Broadway, R.M., Hayes, C.K., Woo, S.L., Noviello, C., Williams, D.L. & Harman, G.E. (1994). Proteinase inhibitors from plants as a novel class of fungicides. *Mol. Plant Microb. Interact.* **7,** 525-527.

Lotan, T., Ori, N. & Fluhr, R. (1989). Pathogenesis-related proteins are developmentally regulated in tobacco flowers. *Plant Cell* **1,** 881-887.

MacIntosh, S.C., Kishore, G.M., Perlak, F.J., Marrone, P.G., Stone, T.B., Sims, S.R. & Fuchs, R.L. (1990). Potentiation of *Bacillus thuringensis* insecticidal activity by serine protease inhibitors. *J. Agric. Food Chem.* **38,** 1145-1152.

Maher, E.A., Bate, N.J., Ni, W., Elkind, Y., Dixon, R.A. & Lamb, C.J. (1994). Increased disease suseptibility of transgenic tobacco plants with suppressed levels of preformed pheynlpropanoid products. *Proc. Natl. Acad. Sci. USA.* **91,** 7802-7806.

Mak, A.S. & Jones, B.L. (1976). The amino acid sequence of wheat β-purothionin. *Can. J. Biochem.* **22,** 835-842.

Marchetti, S., Giordano, A., Chiaba, D., Pfeiffer, A., Delledonne, M., Fogher, C., Savazzni, F., Cervini, P., Bassi, C., Chiesa, F., Reguzzi, M.C. & Cravedi, P. (1994). Genetic engineering of plants for insect resistance using proteinase-inhibitor genes. *Proc. of IPBA, Rogla,* 113-119.

Masoud, S.A., Johnson, L.B., White, F.F. & Reeck, G.R. (1993). Expression of a cysteine proteinase inhibitor (oryzacystain-1) in transgenic tobacco plants. *Plant Mol. Biol.* **21,** 655-663.

McGaughey, W.H. & Whalon, M.E. (1992). Managing insect resistance to *Bacillus thuringiensis* toxins. *Science* **258,** 1451-1455.

McGurl, B., Pearce, G., Orizco-Cardensa, M. & Ryan, C. (1992). Structure, expression and antisense inhibition of the systemin precursor gene. *Science* **255,** 1570-1573.

McManus, M.T. & Burgess, E.P.J. (1995). Effects of soybean (Kunitz) trypsin inhibitor on growth and digestive proteases of larvae of *Spodoptera litura. J. Insect Physiol.* **41,** 731-738.

McManus, M.T., White, D.W.R. & McGregor, P.G. (1994). Accumulation of a chymotrypsin inhibitor in transgenic tobacco can affect the growth of insect pests. *Transgenic Res.* **3,** 50-58.

McPherson, S., Perlak, F., Fuchs, R., MacIntosh, S., Dean, D., Fiskhoff, D. (1989). Expression and analysis of the insect control protein from *Bacillus thuringiensis* var. *tenebrionis.* In: Abstracts of the 1st international symposia of the molecular biology of the potato. Bar Harbour, M.E. pp 51.

Mehan, V.K. & Liao, B.S. (1994). Groundnut bacterial wilt: past, present and future. In: Groundnut bacterial wilt in Asia: Proceedings of the third working group meeting. (Ed.). V.K. Mehan, D. McDonald. ICRISAT, India. pp 67-90.

Mehan, V.K. & McDonald, D. (1994). Groundnut bacterial wilt in Asia: Proceedings of the third working group meeting. ICRISAT, India.

Mendez, E., Moreno, A., Colilla, F., Pelaez, F., Limas, G.G., Mendez, R., Soriano, F., Salinas, M. & de Haro, C. (1990). Primary structure and inhibition of protein synthesis in

eukaryotic cell-free system of a novel, γ-hordothionin, from barley endosperm. *Eur. J. Biochem.* **194,** 533-539.

Métraux, J.P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W. & Inverardi, B. (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* **250,** 1004-1006.

Michaud, D., Berniervadnais, N., Overney, S. & Yelles, S. (1995). Constitutive expression of digestive cysteine proteinase forms during development of the Colorado potato beetle, *Leptinotarsa decemlineata Say* (Coleoptera, Chrysomelidae). *Insect Biochem. Mol. Biol.* **25,** 1041-1048.

Mitsumori, C., Yamagishi, K., Fujino, K. & Kikuta, Y. (1994). Detection of immunologically related Kunitz and Bowman-Birk proteinase inhibitors expressed during potato tuber development. *Plant Mol. Biol.* **26,** 961-969.

Molina, A., Ahl Goy, P., Fraile, A., Sánchez-Monge, R. & García-Olmedo, F. (1993). Inhibition of bacterial and fungal plant pathogens by thionins of types I and II. *Plant Sci.* **92,** 169-177.

Moran, R., Nasuno, S. & Starr, M.P. (1968). Extracellular and intracellular polygalacturonic acid trans-eliminase of *Erwinia carotovora. Arch. Biochem. Biophys.* **123,** 298-306.

Moreno, M., Segura, A. & García-Olmedo, F. (1994). Pseudothionin, a potato peptide active against potato pathogens. *Eur. J. Biochem.* **223,** 135-139.

Mosolov, V.V. & Shul'gin, M.N. (1987). Protein inhibitors of microbial proteinases from wheat, rye and triticale. *Planta* **167,** 595-600.

Mosolov, V.V., Loginova, M.D., Fedurkina, N.V. & Benken, I.I. (1976). The biological significance of proteinase inhibitors in plants. *Plant Sci. Lett.* **7,** 77-80.

Mosolov, V.V., Loginova, M.D., Malova, E.L. & Benken, I.I. (1979). A specific inhibitor of *Colletotrichum lindemuthianum* protease from kidney bean (*Phaseolus vulgaris*) seeds. *Planta* **144,** 265-269.

Mount, M.S., Bateman, D.R., Basham, H.G. (1970). Induction of electrolyte loss, tissue maceration and cellular death of potato tissue by an endopolygalacturonate trans-eliminase. *Phytopathol.* **60,** 924-931.

Murdock, L.L., Shade, R.E. & Pomeroy, M.A. (1988). Effects of E-64, a cysteine proteinase inhibitor, on cowpea weevil growth, development, and fecundity. *Environ. Entomol.* **17,** 467-469.

Narváez-Vásquez, J., Pearce, G., Orozco-Cardenas, M.L., Franceschi, V.R. & Ryan, C.A. (1995). Autoradiographic and biochemical evidence for the systemic translocation of systemin in tomato plants. *Planta* **195,** 593-600.

Nemestothy, G.S. & Guest, D.I. (1990). Phytoalexin accumulation, phenylalanine ammonia lyase activy and ethylene biosythesis in fosetyl-Al treated and resistant and susceptible tobacco cultivars infected with *Phytophthora nicotianae* var. *nicotianae. Physiol. Mol. Plant Pathol.* **37,** 207-219.

Neuhaus, J.M., Ahl-Goy, P., Hintz, U., Flores, U. & Meins, F. (1991). High level expression of a tobacco chitinase gene in *Nicotiana sylvestris:* suseptibility of transgenic plants to *Cecrospora nicotianae. Plant Mol. Biol.* **16,** 141-151.

Newcomb, E.H. (1963). Cytoplasm-cell wall relationships. *Ann. Rev. Plant Physiol.* **14,** 43-64.

Nielsen, K.J., Heath, R.L., Anderson, M.A. & Craik, D.J. (1995). Structures of a series of 6-kDa trypsin inhibitors isolated from the stigma of *Nicotiana alata. Biochem.* **34,** 14304-14311.

Nielsen, K.J., Hill, J.M., Anderson, M.A. & Craik, D.J. (1996). Synthesis and structure determination by NMR of a putative vacuolar targeting peptide and model of a proteinase inhibitor from *Nicotiana alata. Biochem.* **35,** 369-378.

Nordeen, R.O., Sinden, S.L., Jaynes, J.M. & Owens, L.D. (1992). Activity of crecropin SB37 against protoplasts from several plant species and their bacterial pathogens. *Plant Sci.* **82,** 101-107.

Oka, T., Murata, Y., Nakanishi, T., Yoshizumi, H., Hayashida, H., Ohtsuki, Y., Toyoshima, K. & Hakura, A. (1992). Similarity, in molecular structure and function, between the plant toxin purathionin and the mammalian pore-forming proteins. *Mol. Biol. Evol.* **9,** 707-715.

Okada, T. & Yoshizumi, H. (1973). The mode of action of toxic protein in wheat and barley on brewing yeast. *Agric. Biol. Chem.* **37,** 2289-2294.

Okada, T., Yoshizumi, H. & Terashima, Y. (1970). A lethal toxic substance for brewing yeast in wheat and barley. I. Assay of toxicity on various grains, and sensitivity of various yeast strains. *Agric. Biol. Chem.* **34,** 1084-1088.

Olson, T. & Sameulsson, G. (1972). The amino acid sequence of viscotoxin A2 from the European mistletoe (*Viscum album* L., Loranthaceae). *Acta. Chem. Scand.* **26,** 585-595.

Orr, G.L., Strickland, J.A. & Walsh, T.A. (1994). Inhibition of *Diabrotica* larval growth by a multicystatin from potato tubers. *J. Insect Physiol.* **40,** 893-900.

Palm, C.J., Costa, M.A., An, G. & Ryan, C.A. (1990). Wound-inducible nuclear protein binds DNA fragments that regulate a proteinase inhibitor II gene from potato. *Proc. Natl. Acad. Sci. USA.* **87,** 603-607.

Panda, N. & Khush, G.S. (1995). Host plant resistance to insects. CAB International in association with the International Rice Research Institute. Wallingford, Oxon. pp 1-25.

Park, C.M., Berry, J.O. & Bruenn, J.A. (1996). High level secretion of a virally encoded antifungal toxin in transgenic tobacco plants. *Plant Mol. Biol.* **30,** 359-366.

Pautot, V., Holzer, F.M. & Walling, L.L. (1991). Differential expression of tomato proteinase inhibitor I and II genes during pathogen invasion and wounding. *Mol. Plant Microb. Interact.* **4,** 284-292.

Pearce, G., Johnson, S. & Ryan, C.A. (1993). Purification and characterisation from tobacco (*Nicotiana tobaccum*) leaves of six, small, wound-inducible proteinase isoinhibitors of the potato II family. *Plant Physiol.* **102,** 639-644.

Pearce, G., Ryan, C.A. & Liljegren, D. (1988). Proteinase inhibitors I and II in fruit of wild tomato species: Transient components of a mechanism for defense and seed dispersal. *Planta* **175,** 527-531.

Pearce, G., Strydom, D., Johnson, S. & Ryan, C.A. (1991). A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science* **253,** 895-898.

Peferoen, M., Jansens, S., Reynaerts, A. & Leemans, J. (1990). Potato plants with engineered resistance against insect attack. In: Molecular and Cellular Biology of the Potato. (Eds.). M.E. Vayda, M.C. Park. CAB International, Wallingford. pp193-204.

Peña-Cortés, H., Sanchêz-Serrano, J.J., Mertens, R., Willmitzer, L. & Prat, S. (1989). Abscisic acid is involved in the wound-induced expression of the proteinase inhibitor II gene in potato and tomato. *Proc. Acad. Natl. Sci. USA* **86,** 9851-9855.

Peng, J.H. & Black, L.L. (1976). Increased proteinase activity in responsse to infection of resistant tomato plants by *Phytophthora infestans. Phytopathol.* **66,** 958-963.

Perlak, F.J., Deaton, W., Armstrong, R.L., Fushs, R.L., Sims, S.R., Greenplate, J.T. & Fischhoff, D.A. (1990). Insect resistant cotton plants. *Bio/Technology* **8,** 939-943.

Pietrzak, M., Shillito, D., Hohn, T. & Potrykus, I. (1986). Expression in plants of two bacterial antibiotic resistance genes after protoplast transformation with a new plant expression vector. *Nucleic Acids Res.* **14,** 5857.

Piñeiro, M., Diaz, I., Rodriguez-Palenzuela, P., Titarenko, E. & García-Olmedo. F. (1995). Selective disulphide linkage of plant thionins with other proteins. *FEBS Lett.* **369,** 239-242.

Plunkett, G., Senear, D.F., Zuroske, G. & Ryan, C.A. (1982). Proteinase inhibitors I and II from leaves of wounded tomato plants: Purification and properties. *Arch. Biochem. Biophys.* **213,** 463-472.

Ponz, F., Paz-Ares, Hernández-Lucas, C., García-Olmedo, F. & Carbonero, P. (1986). Cloning and nucleotide sequence of a cDNA encoding the precursor of the barley toxin α-hordothionin. *Eur. J. Biochem.* **156,** 131-135.

Ponz, F., Paz-Ares, J., Hernández-Lucas, C., Carbonero, P. & García-Olmedo. F. (1983). Synthesis and processing of thionin precursors in developing endosperm from barley (*Hordeum vulgare* L.) *EMBO J.* **2,** 1035-1040.

Porter, F.M. (1966). Protease activity in diseased fruits. *Phytopath.* **56,** 1424-1425.

Powell, A.L.T., Stotz, H.U., Labavitch, J.M., Bennett. (1994). Glycoprotein inhibitors of fungal polygalacturonases. In: Advances in Molecular Genetics of Plant-Microbe Interactsions. (Ed.) M.J. Daniels. Kluwer Academic Publishers, Netherlands. pp 230-281.

Pueyo, J.J., Hunt, D.C. & Chrispeels, M.J. (1993). Activation of bean (*Phaseolus vulgaris*) α-amylase inhibitor requires proteolytic processing of the proprotein. *Plant Physiol.* **101,** 134-138.

Pusztai, A. (1991). Pea lectins. *Chemistry and Pharmacology of Natural Products Series.* Cambridge University Press, Cambridge.

Raikhel, N.V., Lee, H.I. & Broekaert, W.F. (1993). Structure and function of chitin-binding proteins. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **44,** 591-695.

Raven, P.H. & Johnson, G.B. (1989). Biology, 2nd Edition. Times Mirror, Molsby College.

Reddy, P.V., Lam, C.K., Belanger, F.C. (1996). Mutualistic fungal endophytes express a protease that is homologous to proteases suspected to be important in fungal pathogenicity. *Plant Physiol.* **111,** 1209-1218.

Reimann-Philipp, U., Schrader, G., Martinoia, E., Barkholt, V. & Apel, K. (1989). Intracellular thionins of barley. A second group of leaf thionins closely related to but distinct from cell wall-bound thionins. *J. Biol. Chem.* **264,** 8978-8984.

Reynaerts, A. & Jansens, S. (1994). Engineered resistance in tomato. *Acta Hort.* **376.** In: 5th Annual International Symposia on processing in tomatoes. (Ed.). Bièche, B.J. Sorrento, Italy. pp 347-352.

Rhodes, J.D., Thain, J.F. & Wildon, D.C. (1996). The pathway for systemic electrical signal conduction in the wounded tomato plant. *Planta* **200,** 50-57.

Richardson, M. (1977). The proteinase inhibitors of plants and micro-organisms. *Phytochem.* **16,** 159-169.

Richardson, M. (1991). Seed storage proteins: The enzyme inhibitors. *Methods Plant Biochem.* **5,** 259-305.

Rickauer, M., Fournier, J. & Esquerré-Tugayé, M.T. (1989). Induction of proteinase inhibitors in tobacco cell suspension culture by elicitors of *Phytophthora parasitica* var. *nicotianae. Plant Physiol.* **90,** 1065-1070.

Robin, D. & Guest, D.I. (1994). Characterisation of pathogenicity of *Phytophthora parasitica* isolates by stem and detached-leaf innoculations in four tobacco cultivars. *NZ J. Crop Horticult. Sci.* **22,** 159-166.

Roby, D., Toppan, A. & Esquerré-Tugayé, M.T. (1987). Cell surfaces in plant micro-organism interactions. VIII. Increase in proteinase inhibitor activity in melon plants in response to infection by *Colletotrichum lagenarium* or to treatment with an elicitor fraction from this fungus. *Physiol. Mol. Plant Pathol.* **30,** 453-460.

Rolka, K., Kupryszewski, G., Ragnarson, U., Otlewski, J., Wilusz, T. & Polanowski, A. (1989). Synthesis of an elastase inhibitor by monosubstitution of arginine-5 with valine at the reactive site in a trypsin inhibitor from squash seeds (CMTI III). *Biol. Chem. Hoppe-Seyler* **370,** 499-502.

Roush, R.T. (1994). Managing pests and their resistance to *Bacillus thuringiensis:* Can transgenic plants be better than sprays? *Biocontrol Sci. Technol.* **4,** 501-516.

Rozycki, J., Kupryszewski, G., Rolka, K., Ragnarsson, U., Zbyryt, T., Krokoszynska, I. & Wilusz, T. (1994). Analogues of *Cucurbita maxima* trypsin inhibitor III (CMTI III) with elastase inhibitory activity. *Biol. Chem. Hoppe-Seyler* **375,** 289-291.

Ryan, C.A. (1966). Chymotrypsin inhibitor I from potatoes: reactivity with mammalian, plant, bacterial and fungal proteinases. *Biochem.* **5,** 1592-1596.

Ryan, C.A. (1973). Proteolytic enzymes and their inhibitors in plants. *Ann. Rev. Plant Physiol.* **24,** 173-196.

Ryan, C.A. (1984). Defence Responses of Plants. In: Plant Gene Research: Genes involved in Microbe-Plant Interactions. (Eds.). D.P.S. Verma, T.H. Hohn. Springer-Verlag, Wein, New York. pp 375-386.

Ryan, C.A. (1990). Proteinase Inhibitors in plants: Genes for improving defences against insects and pathogens. *Ann. Rev. Phytopath.* **28,** 425-49.

Ryan, C.A. & An, G. (1988). Molecular biology of wound-inducible proteinase inhibitors in plants. *Plant Cell Environ.* **11,** 345-349.

Ryan, C.A. & Walker-Simmons, M. (1981). Plant Proteinases. In: The Biochemistry of Plants. A comprehensive Treatise **6.** (Eds.). P.K. Stumpf, E.E. Cohn. Academic Press, New York. pp 321-350.

Ryan, C.A., Bishop, P.D., Walker-Simmons, M., Brown, W.E. & Graham, J.S. (1985). In: *UCLA Symposia on Molecular and Cellular Biology, New Series,* **22.** (Eds.). J. Key, T. Kosuge. pp 319-334.

Sall, MA., Teviotdale, B.L. & Savage, S.D. (1982). Bunch Rots. In: Grape pest management. (Eds.). D.L. Flaherty, F.L. Jensen, A.N. Ksimatis, H. Kido, W.J. Moller. Oakland, California, University of California. pp 51-57.

Samuelsson, G. & Pettersson, B. (1971). The amino acid sequence of Viscotoxin B from the European mistletoe (*Viscum album* L., Loranthaceae). *Eur. J. Biochem.* **21,** 86-89.

Sanchêz-Serrano, J.J., Keil, M., O'Connor, A., Schell, J., Willmitzer, L. (1987). Wound-induced expression of a potato proteinase inhibitor II gene in transgenic tobacco plants. *EMBO J.* **6,** 303-306.

Sanchêz-Serrano, J.J., Schmidt, R., Schell, J., Willmitzer, L. (1986). Nucleotide sequence of proteinase inhibitor II encoding cDNA of potato (*Solanum tuberosum*) and its mode of expression. *Mol. Gen. Genet.* **203,** 15-20.

Schägger, H. & Von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analyt. Biochem.* **166,** 368-369.

Schaller, A. & Ryan, C.A. (1994). Identification of a 50 kDa systemin binding protein in tomato plasma membranes having Kex2p-like properties. *Proc. Natl. Acad. Sci. USA* **91,** 11802-11806.

Schaller, A. & Ryan, C.A. (1995). Systemin - a polypeptide defense signal in plants. *Bioessays* **18,** 27-33.

Schrader, G. & Apel, K. (1993). The anticyclic timing of leaf senescence in the parasitic plant *Viscum album* is closely correlated with the selective degradation of sulphur-rich viscotoxins. *Plant Physiol.* **101,** 745-749.

Schrader-Fisher, G. & Apel, K. (1993). cDNA-derived identification of novel thionin precursors in *Viscum album* that contain highly divergent thionin domains but conserved signal and acidic polypetide domains. *Plant Mol. Biol.* **23,** 1233-1242.

Schroeder, H.E., Gollash, S., Moore, A., Tabe L.M., Craig, S., Hardie, D.C., Chrispeels, M.J., Spencer, D. & Higgins, T.J. (1995). Bean α-amylase inhibitor confers resistance to the pea weevil (*Bruchus sativum* L.). *Plant Physiol.* **107,** 1233-1239.

Schroeder, H.E., Gollash, S., Tabe L.M. & Higgins, T.J. (1994). Recent advances in gene transfer to peas. *Pisum Genet.* **26,** 1-5.

Schroeder, H.E., Schotz, A.H., Wardley-Richardson, T., Spencer, D. & Higgins, T.J.V. (1993). Transformation and Regeneration of two cultivars of Pea (*Pisum sativum* L.). *Plant Physiol.* **101,** 751-757.

Schroeder, M.R., Dombrowski, J.E., Bednarek, S.Y., Borkhsenious, O.N. & Raikhel, N.V. (1993). Molecular basis of post-translational modifications and targeting of barley lectin to the vacuoles in barley and in transgenic tobacco plants. *J. Experim. Bot.* **44,** (supplement). 315-319.

Scott, M.P., Jung, R., Muntz, K. & Nielsen, N.C. (1992). A protease responsible for post-translational cleavage of a conserved Asn-Gly linkage in glycinin, the major seed storage protein of soybean. *Proc. Natl. Acad. Sci. USA* **89,** 658-662.

Senser, F., Belitz, H.D., Kaiser, K.P. & Santarius, K. (1974). Suggestion of a protective function of proteinase inhibitors in potatoes: Inhibition of proteolytic activity of microorganisms isolated from spoiled potato tubers. *Z. Lebensm. Unters.-Forsch.* **155,** 100-101.

Shade, R.E., Schroeder, H.E., Pueyo, J.J., Tabe, L., Murdock, L.L., Higgins, T.J.V. & Chrispeels, M.J. (1994). Transgenic pea seeds expressing the α-amylase inhibitor of the common bean are resistant to Bruchid beetles. *Bio/Technology* **12,** 793-796.

Shin, D.H., Lee, J.Y., Hwang, K.Y., Kim, K.K. & Suh, S.W. (1995). High-resolution crystal structure of the non-specific lipid-transfer protein from maize seedlings. *Structure* **3,** 189-199.

Shuckle, R.H. & Murdock, L.L. (1983). Lipoxygenase, trypsin inhibitor and lectin from soybean: effects on larval growth of *Manduca sexta* (Lepidoptera: Sphingidae). *Environ. Ecol.* **12,** 787-791.

Siemens, J. & Schieder, O. (1996). Transgenic plants: genetic transformation - recent developments and state of the art. *Plant Tissue Culture Biotech.* **2,** 66-75.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. & Klenk, D.C. (1985). Measurement of protein using bicinochonic acid. *Analyt. Biochem.* **150,** 76-85.

Stirpe, F. & Hughes, R.C. (1989). Specificity of ribosome-inactivating proteins with RNA *N*-glycosidase activity. *Biochem. J.* **262,** 1001-1002.

Stirpe, F., Barbieri, L., Battelli, M.G., Soria, M. & Lappi, D.A. (1992). Ribosome-inactivating proteins from plants: present status and future prospects. *Bio/Technology* **10,** 405-412.

Stuart, L.S. & Harris, T.H. (1942). Bactericidal and fungicidal properties of a crystalline protein islated from unbleached wheat flour. *Cereal Chem.* **19,** 288-300.

Tabe, L.M., Higgins, C.M., McNabb, W.C. & Higgins T.J.V. (1993). Genetic engineering of grain and pasture legumes for improved nutritive value. *Genetica* **90,** 181-200.

Tabe, L.M., Wardley-Richardson, T., Ceriotti, A., Aryan, A., McNabb, W., Moore, A. & Higgins, T.J.V. (1995). A biotechnological approach to improving the nutritive value of alfalfa. *J. Anim Sci.* **73,** 2752-2759.

Taylor, B.H., Young, R.J., Scheruing, C.F. (1993). Induction of a proteinase inhibitor II-class gene by auxin in tomato roots. *Plant Mol. Biol.* **23,** 1005-10014.

Teeter, M.M., Mazer, J.A. & L'Italien, J.J. (1981). Primary structure of the hydrophobic plant protein crambin. *Biochem.* **20,** 5437-5443.

Terras, F.R.G., Eggermont, K., Kovaleva, V., Kaikhel, N.V., Osborn, R.W., Kester, A., Rees, S.B., Torrekens, S., Van Leuven, F., Vanderleyden, J., Cammue, B.P.A. & Broekaert, W.F. (1995). Small cysteine-rich antifungal proteins from radish: Their role in host defence. *Plant Cell.* **7,** 573-588.

Terras, F.R.G., Goderis, I.J., Van Leuven, F., Vanderleyden, J., Cammue, B.P.A. & Broekaert, W.F. (1992a). *In vitro* antifungal activity of a radish (*Raphanus sativus* L.) seed protein homologous to nonspecific lipid transfer proteins. *Plant Physiol.* **100,** 1055-1058.

Terras, F.R.G., Schoofs, H.M.E., De Bolle, M.F.C., Van Leuven, F., Rees, S.B., Vanderleyden, J., Cammue, B.P.A. & Broekaert, W.F. (1992b). Analysis of two novel classes of plant antifungal peptides from radish (*Raphanus sativus* L.) seeds. *J. Biol. Chem.* **267,** 15301-15309.

Terras, F.R.G., Schoofs, H.M.E., Thevissen, K., Osborn, R.W., Vanderleyden, J., Cammue, B.P.A. & Broekaert, W.F. (1993a). Synergistic enhancement of the anti-fungal activity of wheat and barley thionins by radish and oilseed rape 2S albumins and by barley trypsin inhibitors. *Plant Physiol.* **103,** 1311-1319.

Terras, F.R.G., Torrekens, S., Van Leuvan, F., Osborn, R.W., Vanderleyden, J., Cammue, B.P.A. & Broekaert, W.F. (1993b). A new family of cysteine-rich plant antifungal proteins from Brassiceae species. *FEBS Lett.* **316,** 233-240.

Thomas, J.C., Adams, D.G., Keppenne, V.D., Wasmann, C.C., Brown, J.K., Kanost, M.R. & Bohnert, H.J. (1995a). *Manduca sexta* encoded protease inhibitors expressed in *Nicotiana tabacum* provide protection against insects. *Plant Physiol. Biochem.* **33,** 611-614.

Thomas, J.C., Adams, D.G., Keppenne, V.D., Wasmann, C.C., Brown, J.K., Kanost, M.R. & Bohnert, H.J. (1995b). Protease inhibitors of *Manduca sexta* expressed in transgenic cotton. *Plant Cell Rep.* **14,** 758-762.

Thompson R. (1980). Maximum likelihood estimation of variance components. *Math. Operationsforsch. Statsit., Ser. Statist.* **11,** 545-561.

Thornburg, R.W., An, G., Cleveland, T.E., Johnson, R. & Ryan, C.A. (1987). Wound inducible expression of a potato inhibitor II-chloramphenicol acetyltransferase gene fusion in transgenic tobacco plants. *Proc. Natl. Acad. Sci. USA.* **84,** 744-748.

Vaeck, M., Reynaerts, A., Höfte, H., Jansens, S., De Beuckeleer, M., Dean, C., Zabeau, M., Van Montagu, M. & Leemans, J. (1987). Transgenic plants protected from insect attack. *Nature* **328,** 33-37.

van den Elzen, P.J.M., Jongedijk, E., Melchers, L.S. & Cornelissen, B.J.C. (1993). Virus and fungal resistance: from laboratory to field. *Phil. Trans. Royal Soc. London B.* **342,** 271-278.

van Emden, H.F., Ball, S.L. & Rao, M.R. (1988). Pest, disease and weed problems in pea, lentil, fabia bean and chickpea. In: World Crops: Cool season food legumes. (Ed.). R.J. Summerfield. Kluwer Academic Publishers, Netherlands. pp 519-534.

van Etten, C.H., Gagne, W.E., Robins, D.J., Booth, A.N., Daxenbichler, M.E. & Wolff, I.A. (1969). Biological evaluation of *Crambe* seed meals and derived products by rat feeding. *Cereal Chem.* **46,** 145-155.

van Rie, J. (1991). Insect control with transgenic plants: resistance proof? *Trends Biotechnol.* **9,** 177-179.

Vermeulen, J.A.W.H., Lamerichs, R.M.J.N., Berliner, L.J. De Marco, A., Llinás, M., Boelens, R., Alleman, J. & Kaptein, R. (1987). ^{1H}NMR characterization of two crambin species. *FEBS Lett.* **219,** 426-430.

Vernon, L.P. & Rogers, A. (1992). Effect of calcium and phosphate ions on hemolysis induced by *Pyrularia* thionin and *Naja naja kaouthia* cardiotoxin. *Toxicon.* **30,** 211-721.

Vernon, L.P., Evett, G.E., Zeikus, R.D. & Gray, W.R. (1985). A toxic thionin from *Pyrularia pubera:* purification, properties and amino acid sequence. *Arch. Biochem. Biophys.* **238,** 18-29.

Vock, N.T. (1978). A handbook of plant diseases in colour. *Queensland Department of Primary Industries, Plant Pathology Branch.* **2,** 26.

Wada, K. & Buchanan, B.B. (1981). Purothionins: a seed protein with thioredoxin activity. *FEBS Lett.* **124,** 237-240.

Wallis, F.M. & Truter, S.J. (1978). Histopathology of tomato plants infected with *Pseudomonas solanacearum,* with emphasis on ultrastructure. *Physiol. Plant Pathol.* **13,** 307-317.

Ward, E., Uknes, S. & Ryals, J. (1994). Molecular biology and genetic engineering to improve plant disease resistance. In: Molecular biology in crop protection. (Eds.). G. Marshall, D, Walters. Chapman & Hall, London. pp 121-145.

Wildon, D.C., Doherty, H.M., Eagles, G., Bowles, D.S. & Thain, J.F. (1989). Long range electrical signals. *Ann. Bot.* **646,** 691-695

Wildon, D.C., Thain, J.F., Minchin, P.E.H. (1992). Electric signaling and systemic proteinase inhibitor induction in the wounded plant. *Nature* **360,** 62-65.

Willmitzer, L. (1988). The use of transgenic plants to study plant gene expression. *Trends Genet.* **4,** 13-18.

Wolfson, J.L. & Murdock, L.L. (1987). Supression of larval Colorado potato beetle growth and development by digestive proteinase inhibitors. *Entomol. Exp. Appl.* **44,** 235-240.

Wu, G., Shortt, B.J., Lawrence, E.B., Levine, E.B., Fitzsimmons, K.C. & Shah, D.M. (1995). Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. *Plant Cell* **7,** 1357-1368.

Xu, D., Qingzhong X., McElroy, D., Mawal, Y., Hilder, V.A. & Wu, R. (1996). Constitutive expression of a cowpea trypsin inhibitor gene, CpTI, in transgenic rice plants confers resistance to two major rice insect pests. *Molec. Breeding* **2,** 167-173.

Xu, G. & Qin, J. (1994). Extraction and characterization of midgut proteases from *Heliothis armigera* and *H. assulta* (Lepidoptera: Noctuidae) and their inhibition by tannic acid. *J. Econ. Entomol.* **87,** 334-338.

Zalucki, M.P., Daglish, G., Firempong, S. & Twine, P. (1989). The biology and ecology of *Helicoverpa armigera* (Hübner) and *Helicoverpa punctigera* Wallengren (Lepidoptera: Noctuidae) in Australia: What do we know? *Aust. J. Zool.* **34,** 779-814.

Zambryski, P. (1988). Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. *Ann. Rev. Genet.* **22,** 1-30.